© 2017 Jennifer Williams

EFFECTS OF 2′-FUCOSYLLACTOSE ON BRAIN AND COGNITIVE DEVELOPMENT OF INTRAUTERINE GROWTH RESTRICTED PIGLETS

BY

JENNIFER NICOLE WILLIAMS

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

Master's Committee:

Professor Kelly S. Swanson, Chair
Professor Rodney W. Johnson, Director of Research
Assistant Professor Hannah D. Holscher

ABSTRACT

Intrauterine growth restriction (IUGR) leads to small for gestational age (SGA) neonates, increasing risk of brain and cognitive impairments that persist into adulthood. Human milk oligosaccharides (HMO) have been postulated to exert positive effects on brain development. HMOs are the third most abundant solid component of breast milk with 2′-fucosyllactose (2′FL) being the most abundant HMO. We aimed to determine whether orally administered 2′FL enhances brain and cognitive development in small for gestational aged neonates. Sex-matched, littermate pairs of SGA (0.5-0.9kg) and appropriate for gestational age (AGA, 1.3-1.8kg) piglets were acquired on postnatal day (PD) 2, placed in individual cages, and divided into two diet groups. One group was provided a sow milk replacer diet (300ml/kg/d) while the other received the same diet supplemented with 2′FL (1.17g/L). On PD 14, spatial learning was assessed using a T-maze task, and on PD 28, brain macrostructure, microstructure, and hippocampal metabolites were assessed by magnetic resonance imaging. A sub-set of pigs was euthanized at PD14 and hippocampal tissue was dissected and used for RNA-sequencing. Body weight was different between SGA and AGA throughout the course of the study ($p<0.05$), with 2′FL having no effect. At PD 28, the brain-to-body-weight ratio of SGA piglets was greater than that of AGA piglets ($p<0.0001$), suggesting a "brain-sparing" effect during IUGR. The brain-to-body-weight ratio was not affected by 2′FL. Performance of all piglets during acquisition and reversal phases of the spatial T-maze task improved over time ($p<0.0001$), but SGA piglets reached criterion faster than AGA piglets ($p<0.05$), with no effect of diet. MRI revealed 12 brain regions ($p<0.01$) that were larger in AGA than SGA piglets. Furthermore, SGA piglets had less grey matter than AGA ($p<0.001$) in 4 subcortical regions and reduced whole brain fractional anisotropy ($p<0.01$) suggesting lower white matter integrity. No effects of 2′FL on brain structure were found. RNA-

sequencing revealed 529 differentially expressed genes (DEGs) in hippocampal tissue ($p<0.006$). A total of 115 DEGs were identified in response to size, of which 62 were up-regulated and 53 were down-regulated in SGA piglets compared to AGA piglets. Up-regulated genes in SGA piglets were associated with chromatin silencing, DNA binding, nuclear chromatin, and the nucleosome; and down-regulated genes in SGA piglets were associated with KEGG pathways for measles, influenza A, hepatitis C, and herpes simplex infection. A main effect of diet revealed 252 DEGs, of which 144 up-regulated and 108 down-regulated genes in 2′FL piglets compared to CON. Genes that were up-regulated in response to 2′FL were connected to integral components of membranes and transmembrane helix, while down-regulated genes were clustered based on ion channel and transport, zinc ion binding, zinc-finger, and metal-binding. Finally, in the size by diet interaction, there were no clusters discovered as a result of up-regulated gene expression, however, down-regulated genes were associated with transmembrane helix, membranes, and integral components of membrane. Collectively, the results show that neonates born SGA have altered brain structure and hippocampal gene expression. While 2′FL did not have measureable effects on brain structure or spatial learning in either SGA or AGA piglets, it elicited a distinct gene expression profile in the hippocampus suggesting epigenetic modifications that may contribute to development and function.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

Intrauterine growth restriction (IUGR) often leads to small for gestational age (SGA) neonates, increasing the risk of perinatal morbidity and mortality (1). IUGR can be defined as impaired growth and development of an embryo/fetus or its organs during pregnancy (2) and is observed in approximately 24% of newborn human infants annually (3). An infant is classified as SGA if it has a birth weight in the lower 10^{th} percentile for its gestational age (4). While IUGR and SGA are associated with several health problems throughout life, studies have shown a connection with neurodevelopmental disabilities in cognitive function, recognition memory, attention, mood, and school performance (5, 6).

Robust growth and expansion of the brain occurs during the last trimester and the first year of life (7, 8). During this period, the brain is vulnerable to insults that may be mitigated with proper early-life nutrition. One brain region particularly susceptible to these perinatal stressors is the hippocampus, a region involved in learning and memory (9). These deficits often persist into adulthood (10), and therefore, it is imperative to develop efficacious interventions for reversing or mitigating the cognitive impairments associated with being born SGA due to IUGR.

Human milk is commonly referred to as the "gold standard" of infant nutrition. It contains all of the essential nutrients for the growth and development of the infant as well as provides infectious disease protection (11-13) and has been shown to improve cognitive development (14, 15). In addition to the traditional nutrients, breast milk contains a variety of functional ingredients that may provide a health benefit to the infant (16). One of these functional ingredients are human milk oligosaccharides (HMOs). HMOs are the third most abundant solid component of breast milk and contain a range of structurally diverse unconjugated glycans (17).

There have been a variety of postulated beneficial effects for HMOs. Research suggests that HMOs act as antiadhesive antimicrobials that serve as soluble decoy receptors to prevent pathogen adhesion (17). Furthermore, they regulate epithelial and immune cell responses (18), regulate lymphocyte cytokine production (17), and modulate the intestinal microbiota (16, 17, 19). While there are approximately 200 different complex oligosaccharides (16), this study focuses on 2′-fucosyllactose (2′FL), the most abundant HMO. A growing body of evidence suggests 2′FL has anti-infective properties, acts as a prebiotic to feed beneficial bacteria (20), and enhances cognitive development (21-23).

Understanding the influence of 2′FL and IUGR on brain and cognitive development has been slow due to obvious ethical considerations when studying human infants; therefore, the neonatal piglet appears to be an excellent model. In regards to neurodevelopment, the piglet has many similarities to the perinatal growth pattern and structure of the human brain (24, 25). Furthermore, the distribution of grey and white matter of the neonatal piglet brain is similar to that of human infants (26). In pigs, IUGR is naturally occurring and often caused by placental insufficiency and multifetal pregnancy (27). In humans, it is often difficult to tell gestational age, but because artificial insemination is used in our system and the time of conception is known, the effects of being born SGA can be differentiated from the effects of being born preterm and low birth weight (27). Thus, the SGA piglet is an excellent model for assessing the neurological effects of nutritional interventions in SGA infants during the neonatal period. This study aims to determine whether orally administered 2′FL enhances hippocampal-dependent learning and memory in small for gestational aged infants using a neonatal piglet model.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRAUTERINE GROWTH RESTRICTION (IUGR), SMALL FOR GESTATIONAL AGE (SGA) AND APPROPRIATE FOR GESTATIONAL AGE (AGA)

2.1.1 Definition of IUGR, SGA, AGA

Intrauterine growth restriction (IUGR) occurs when an infant fails to attain his or her in utero growth potential (28). This can have a lifelong influence on the neonates potential for development and survival. Small for gestational age (SGA) neonates often result from IUGR. An infant is classified as SGA if it has a birth weight in the lower 10^{th} percentile for its gestational age (4). In humans, it is often difficult to determine the gestational age of a neonate because the time of conception is not always known. Using these definitions, all IUGR infants can be categorized as SGA, but not all SGA infants will be IUGR.

The SGA infant may be symmetrically or asymmetrically small at birth compared to its appropriate for gestational age (AGA) counterpart. Symmetrically growth restricted infants have overall reduced weight, length, and head circumference at birth (28). Infants with asymmetric growth restriction typically have spared head growth relative to weight and length (28). Yet, both types of SGA infants have increased risk of perinatal morbidity and mortality, and this risk increases with the decrease in birth weight percentile (29). Due to the fact that many clinical studies regarding IUGR infants often include those that are SGA due to other reasons, these terms will be used interchangeably in this review.

2.1.2 Causes of IUGR and SGA

It is important for clinicians to determine the causes of the growth restriction in order to develop efficacious interventions in reducing the incidence or severity of the condition. The

causes of IUGR are diverse, and poor fetal growth often reflects a combination of maternal, fetal, and placental factors. Maternal conditions associated with IUGR include medical complications (e.g. hypertension, diabetes, severe chronic infection), ethnicity, pre-pregnancy weight and pregnancy weight gain, and low maternal age (4). Epidemiological research has also shown a connection between environmental factors such as smoking, alcohol, high altitude, and low socioeconomic status and incidence of IUGR (4). Among those factors, cigarette smoking appears to be the most influential cause of IUGR. Moreover, fetal conditions associated with IUGR include genetic factors (i.e. chromosomal abnormalities and mutations), infections, and malformations such as cardiovascular, gastrointestinal, and skeletal defects (4). IUGR can also be caused by placental insufficiency. This is a pathological condition that results in the poor transfer of nutrients and potentially oxygen to the fetus (30). IUGR is observed in approximately 24% of newborn human infants annually (3); therefore, it is important to understand the pathophysiology of this condition.

2.1.3 Effects of SGA and IUGR on gastrointestinal development

Infants born SGA are subjected to feeding intolerance, lower fat absorption, and digestive diseases early in life (31). Research has shown changes in the intestines of IUGR neonates including reduced weight, length, wall thickness, villus height, and crypt depth (32). Furthermore, SGA infants show lower efficiency of nutrient utilization when compared to their AGA counterparts (33). Yet, due to ethical considerations, the pathophysiology of growth restriction on the gastrointestinal tract has been difficult to investigate in human infants. Therefore, animal models have been utilized to help advance the knowledge IUGR and its effect on gastrointestinal development.

A recent study explored the effects of IUGR on small intestinal mucosal permeability using a neonatal piglet model (33). FITC-dextran 4 and horseradish peroxidase fluxes, histomorphologic measurements, and gene expression of tight-junction proteins were used to assess epithelial barrier function. The results showed that intestinal permeability was 2-fold higher in the proximal intestine on postnatal day 0 in IUGR piglets. This suggests a compromised barrier function, which could be detrimental to the health of IUGR neonates. A disrupted barrier function could allow the translocation of antigens and/or bacteria leading to inflammatory responses.

Another study using a neonatal piglet model aimed to determine the effects of IUGR on intestinal structure and function (34). Control or IUGR newborn piglets were delivered by cesarean section at full term or premature (91% gestation). While organ weights and intestinal enzyme activities were not notably affected by IUGR for term or pre-term piglets, IUGR piglets were associated with a relatively long and thin intestine. Moreover, IUGR piglet ileum appeared to be developmentally delayed such that they had lower ileal density and villus area, higher expression of the peptide transporter PEPT1, and enhanced bacterial adhesion and translocation. The elevated expression of PEPT1 could potentially result in a decreased barrier function enhancing intestinal transport of bacterial peptides. Taken together, these studies further develop our understanding of the IUGR neonate's increased risk of perinatal morbidity and mortality.

2.1.4 Effects of SGA and IUGR on neurodevelopment

Epidemiological research has shown that SGA and IUGR neonates have an increased risk of cognitive impairments that persist into adulthood. A 26-year follow up study of the 1970 British Birth Cohort aimed to determine the long-term functional outcome of SGA infants (35).

School performance and achievement were assessed at ages 5, 10, and 16 years, and years of education, occupational status, income, marital status, life satisfaction, disability, and height were measured at 26 years in a total of 14,189 full-term infants (1,064 SGA). The results at 5, 10, and 16 years showed that SGA neonates displayed small but significant deficits in academic achievement. For example, teachers were more likely to recommend special education and less likely to rank those born SGA in the top 15th percentile of the class at 16 years. Yet, at age 26, those born SGA did not present any differences in years of education, employment, marital status, or life satisfaction. However, they were less likely to hold managerial or professional jobs as well as have lower levels of weekly income. Collectively, these results show that adults born SGA had significant differences in academic and professional achievement compared to AGA adults. Nevertheless, there were no notable social or emotional consequences of being born SGA at age 26.

Another study followed a total of 7,388 term infants from birth to 14 years to determine if the presence, severity, or symmetry of growth restriction is a risk factor for learning, cognitive, and attention problems in adolescence (36). The results showed that adolescents born SGA were more likely to experience learning difficulties, as measured using the Wide Range Achievement Test (WRAT) reading subtest. However, using the Ravens IQ test, there was no significant difference between SGA and AGA groups for either male or female children. Furthermore, there was no connection between body symmetry and any of the outcomes studied. Thus, it was concluded that SGA status at birth has slight effects on learning, cognition, and attention in adolescence. These learning difficulties were predicted by the severity of SGA, not the symmetry.

2.2 NUTRITION FOR THE NEONATE

2.2.1 Human milk (HM) vs. infant formula (IF)

Human milk is commonly referred to as the "gold standard" of infant nutrition. The American Academy of Pediatrics (AAP) recommends that infants be exclusively breastfed for the first six months of life (37). Solid foods are to be gradually introduced into the infant's diet in addition to breastfeeding until the infant is one year old. According to the latest National Immunization Survey data, in the US, 4 out of 5 (81.1%) infants are breastfed immediately after birth (38). However, this percentage drops significantly by six months to 51.8%, and only about one third (30.7%) of infants are breastfeeding at 12 months (38). At this time, mothers are either relying entirely on infant formula or they are supplementing breast milk with formula. The health benefits of breastfeeding have long been recognized (39). Evidence suggests human milk provides infectious disease protection (11-13), aids in neurodevelopment (14, 15, 39), and improves developmental outcomes of premature and low birth weight infants (40). While infant formula can never replace breast milk, for mothers that need or choose to use it, it is important to narrow the gap between the two in order to ensure proper neonatal nutrition.

2.2.2 Milk components: HM vs. IF

While infant formula has made tremendous strides in becoming more similar to human milk, it is important to note the differences between the two. The composition of human milk is influenced by maternal, environmental, and genetic factors, leading it to be quite variable (41). The composition of human milk can change within a feeding, diurnally, and over the course of lactation in contrast to the standardized compositional range of infant formula (42). It is distinctly suited for the infant, in both its nutritional composition and bioactive factors that aid in

healthy neonatal development (42). Furthermore, human milk contains active enzymes to enhance maturation of the gut and anti-infective properties that protect the newborn from infection (43). While human milk is superior to infant formula in many respects, formula can still promote efficient growth, development, and nutrient balance of the infant (44).

2.2.3 Human milk oligosaccharides

Human milk oligosaccharides (HMOs) are the third most abundant solid component of breast milk and contain a range of structurally diverse unconjugated glycans (17). One liter of mature human milk contains 10-15 g HMO, and concentrations in colostrum (the first milk released from the breast) are even more abundant (45). While over 200 HMOs have been identified, their composition varies from person to person and throughout the course of lactation (46). HMO patterns in breast milk depend on the genetic background of the mother, i.e., on the Lewis blood group and secretor status (47). Once ingested by the infant, HMOs withstand the low pH of the gut and resist degradation by pancreatic enzymes (48). Thus, intact HMOs pass through the distal ileum and serve as nutrients for colonic bacteria.

Originally, HMOs were thought to promote the health outcome of the neonate by serving as a prebiotic that feeds the beneficial bacteria of the gut (17). However, a growing body of evidence suggests HMOs are more than just “food for bugs.” Many bacterial, viral, and even protozoan pathogens need to adhere to mucosal surfaces to invade and cause disease. Some HMOs serve as soluble decoy receptors by resembling mucosal cell surface glycans to prevent pathogen binding (17). Furthermore, HMOs have been associated with other biological processes including anti-inflammatory properties, prevention of necrotizing enterocolitis, and enhancement of brain development (49). Sialylated HMOs have been identified as modulators of anxious

behavior (50), while fucosylated HMOs have been linked to enhancing learning and memory in rodents (21-23).

2.3 2′-FUCOSYLLACTOSE

2.3.1 Metabolism and function

2′-fucosyllactose (2′FL) is the most abundant HMO in human milk with a concentration of up to 4.65 g/L (49). The fucosylation of HMOs is determined by the mother's histo-blood group antigen status, particularly the Secretor and Lewis groups (51). It is synthesized in vivo by a fucosylation reaction catalyzed by α-(1,2)-fucosyltransferase (FucT2) using GDP-L-fucose as a fucose donor substrate to lactose (52). The addition of the monosaccharide, fucose, to lactose renders the resulting trisaccharide resistant to digestion by human enzymes (20). This allows 2′FL to gain access to the colon where it can either by fermented by resident bacteria or excreted in the feces. Recent research has also shown the detection of 2′FL in the urine and plasma of both breast-fed infants and those fed formula supplemented with 2′FL (51, 53). This suggests that 2′FL can be absorbed and reach systemic circulation. Over the last decade, researchers have begun looking into the metabolic functions of 2′FL.

2.3.2 Effect on gut microbiota

It has been well established that the gut microbiota of the breastfed infant is vastly different than those fed infant formula (54-57). Specifically, the microbiome of breast-fed infants is often rich in *Bifidobacterium* species (58). Maternal fucosyltransferase 2 status has been shown to affect gut bifidobacteria communities of breastfed infants (59), suggesting a connection between 2′FL and bifidobacteria. *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium*

bifidum, and *Bifidobacterium breve* possess the glycosyl hydrolase fucosidase that metabolize 2′FL (59). This can be beneficial to the infant due to bifidobacteria's association with protection from pathogens, improved immune response to vaccines, and development of the neonatal immune system (59). Moreover, a recent study looked at the utilization of 2′FL by isolated human gut microbes. The results showed that *Bifidobacterium* spp. and *Bacteroides* spp. exhibited a significant growth increase when 2′FL was added to the culture media (60). In contrast, 2′FL induced minimal growth of *Lactobacillus delbrueckii* ATCC7830, *Enterococcus faecalis* ATCC19433, and *Streptococcus thermophiles* ATCC19258. These data illustrate that 2′FL may protect the neonate by enhancing the growth of commensal bacteria while inhibiting the growth of pathogens.

2.3.3 Effect on immune development

Recent research has shown that infants fed formula supplemented with 2′FL have an immune response similar to those that are breastfed. Using biomarkers of immune function, a randomized, double blind clinical study compared 200 babies exclusively breastfed, formula fed with 2′FL, or formula fed without 2′FL (61). The results showed that infants fed formula containing 2′FL exhibit lower plasma and ex vivo inflammatory cytokine profiles much like breastfed babies. Furthermore, 2′FL has been shown to suppress CD14 expression and release of IL-8 in human enterocytes, reducing inflammation induced by bacterial invasion (62). The neonate is vulnerable, particularly to bacterial infection; this finding further illustrates 2′FL as an important contributor to the infant's developing immune system.

2′FL has been shown to inhibit binding and infection of several specific enteropathogens (63). As their initial step of invasion, many human pathogens bind to mucosal surface receptors

that terminate in α1,2-linked fucose (63). This allows 2′FL to protect the infant from infection by acting as a decoy receptor to competitively inhibit pathogen binding. Moreover, 2′FL has been shown to have some therapeutic potential in allergy symptomology and immune response. Using an ovalbumin-sensitized mouse model of food allergy, the effects of oral treatment with 2′FL on anaphylactic symptoms were investigated (64). The results from this study showed 2′FL diminished food allergy symptoms (e.g. diarrhea, hypothermia) and the passive cutaneous anaphylaxis response.

2.3.4 Effect on brain and cognitive development

It has been proposed that HMOs exert positive effects on the brain and cognitive development of infants. However, most of the research has focused on sialylated structures, and the effects of 2′FL need to be further elucidated. In a recent study, 2′FL supplementation during lactation improved learning and memory in both young and adult rats (22). These cognitive functions were assessed by behavioral and electrophysiological measurements right after weaning and again during adulthood. While the effects of early-life exposure of 2′FL on the behavior of young animals were not detectable, there was a benefit to older animals. In a novel object recognition task, adult rats fed 2′FL during lactation spent significantly longer time examining the novel object rather than exploring the familiar one. It is possible that the behavioral tests used in this study were not sensitive enough to detect differences in the young rats, so an electrophysiological technique (i.e. hippocampal long-term potentiation, LTP) to test synaptic function was used. The results showed a positive effect of 2′FL on LTP in both youth and adulthood.

The same group was then interested in understanding if this effect of 2′FL on the brain was through the gut-brain axis, specifically the vagal nerve (23). Rats were submitted to bilateral subdiaphragmatic vagotomy and orally administered 2′FL through the diet. The results showed that chronic oral administration of 2′FL improves LTP, but these effects were inhibited in vagotomized rats. Furthermore, rats were trained in Skinner boxes, an operant conditioning task, to press a lever to obtain a small pellet of food. While all experimental animals acquired the task, the 2′FL non-vagotomized rats reached criterion in less sessions than all other groups. They also presented a better performance (i.e. higher mean number of lever presses per session) than the other three groups. These effects were eliminated by the bilateral vagotomy. This suggests that the modulatory effects of 2′FL on brain function and cognition could be due to the gut-brain axis, but more specifically, the vagal nerve.

2.4 THE PIG AS A MODEL FOR HUMANS

The neonatal piglet is an emerging model for pediatric biomedical research and often used in studies that would be unethical to attempt in humans. Within the last few decades, the pig has been utilized to study cardiovascular physiology, obesity, stress, dermatology, immunology, behavior, as well as many aspects of nutrition (65). The anatomy and physiology of the digestive tract of the pig is much more similar to humans than rodents. In particular, the rodent is a hindgut fermenter and relies on cecal fermentation. Like humans, the pig possesses a monogastric digestive tract that relies on both cecal and colonic fermentation. Piglet metabolism has been extensively studied (66), and thus the abundance of information increases the reliability of the piglet as a model for pediatric nutrition. Furthermore, the biology of the pig shows strong

similarities with human brain anatomy and perinatal growth patterns (67) making it a useful model for studying neurodevelopment in human infants.

Recently, the neonatal piglet has been established as a model for studying the effects of intrauterine growth restriction on neurodevelopment (27). In pigs, IUGR is naturally occurring, and is often linked to placental insufficiency and litter size (2). Some "runts" are born almost one half to one third the size of their largest littermates (68). For both species, IUGR often leads to SGA neonates. Yet, in humans, it is difficult to distinguish the effects of being born SGA from being born preterm or low birth weight (LBW). Determining gestational age in humans can be challenging (69). However, in swine production, artificial insemination is used, and therefore the time of conception is known. Thus, SGA piglets are a useful model for human infants born SGA due to IUGR.

2.5 SUMMARY

Robust growth and expansion of the brain occurs during the last trimester and the first year of life (7, 8). The brain is particularly vulnerable during this period, and long-lasting, potentially permanent, harm on brain structure and function can occur if these early developmental processes are disrupted. One region highly susceptible to stressors during this time is the hippocampus, an important brain region involved in learning and memory. Intrauterine growth restriction can have a lifelong influence on the neonates potential for development and survival. These deficits often persist into adulthood (10), and therefore, it is imperative to develop efficacious interventions for reversing or mitigating the cognitive impairments associated with being born SGA due to IUGR. Human milk is commonly referred to as the "gold standard" of infant nutrition for it contains all of the essential nutrients for the

growth and development of the infant**.** While infant formula can never replace breast milk, for mothers that need or choose to use it, it is important to narrow the gap between the two in order to ensure proper neonatal nutrition.

CHAPTER THREE

METHODS

3.1 ANIMALS, HOUSING, AND DIETS

Sex-matched, littermate pairs of naturally farrowed piglets (AGA, 1.3-1.8kg; SGA, 0.5-0.9kg) were obtained from the University of Illinois swine herd. Piglets were transported to the biomedical animal facility 48 h after birth (to allow for adequate colostrum intake) and received an intramuscular injection of iron dextran (100mg; Henry Schein, Dublin, OH, USA) and a broad-spectrum antibiotic (Gentamicin Piglet Injection, 5mg; AgriLabs, St. Joseph, MO, USA), which is standard industry practice for animals of this age. The piglets were individually housed in clear, acrylic-sided cages (0.75 m long X 0.58 m wide X 0.47 m high), as previously described (70, 71). Each cage was positioned in a rack and fitted with flooring designed for neonatal animals (Tenderfoot/NS®, Tandem Products, Inc., Minneapolis, MN, USA) to separate them from their excrement. A cotton towel and toy (plastic Jingle Ball™, Bio-Serv, Frenchtown, NJ, USA) were provided for enrichment. Room temperature was maintained at 27°C and radiant heaters located directly above piglets provided supplemental heat. A 12-h light/dark cycle was maintained; however, minimal lighting was provided during dark cycle.

Piglets were assigned to 4 treatment groups controlling for body weight (BW), sex, and litter of origin (n = 9 per group): Control-AGA, Control-SGA, 2′FL-AGA, and 2′FL-SGA. Piglets were fed a nutritionally adequate milk replacer diet (Advance Liqui-Wean, Milk Specialties Co., Dundee, IL, USA) upon arrival at the animal care facility. Milk was reconstituted each morning to a final concentration of 206 g/L using tap water and fed at a rate of 300 ml/kg body weight (based on daily recorded weights). 2′FL was orally administered through the diet at a concentration of 1.17 g/L. The dose of 2′FL was calculated so that piglets received

approximately 350 mg 2′FL/kg of body weight (BW) per day. This is roughly equivalent to the average dose a breast fed infant would receive. No extra water was provided other than that used in milk replacer. Meals were provided in bowls located in the home cage after cognitive testing and every 3 h thereafter. Piglets were fasted for a 10-h period prior to the start of cognitive testing to ensure motivation for milk rewards used in task. All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

3.2 COGNITIVE TESTING

Piglet spatial learning and memory was assessed using a clear plastic plus-shaped maze (double T-maze) with extra-maze visual cues, as previously described and validated (72). The maze consisted of 2 start arms (north and south) and 2 reward arms (east and west). Start arm location was randomized throughout testing to force piglets to rely on a hippocampal-dependent allocentric mechanism (i.e. create spatial map of room using extra-maze visual cues) instead of a striatum-dependent egocentric mechanism (i.e. turn body left or right) for solving the task (73). Starting on postnatal day (PD) 14, piglets were tested each morning between 08:00 h and 12:00 h by one trained experimenter following overnight food deprivation. Testing consisted of 10 trials per day (60 s per trial) for 10 days. The first 6 days involved the acquisition phase of testing, where piglets learned to locate the chocolate milk reward (3 ml of control milk replacer used for daily feedings with addition of Nesquik cocoa powder, supplied according to manufacturer's directions) in a constant place and direction (e.g., east reward arm). To balance for olfactory cues, chocolate milk was provided in both reward arms, but was only accessible in the correct

arm. This was followed by 4 days of the reversal phase, where the previously incorrect arm (e.g., west reward arm) was now rewarded. A learning criterion of 80% correct was established for both the acquisition and reversal phases of testing. Piglet movement within the maze was tracked live using commercially available software (EthoVision 3.1; Noldus Information Technology Inc., Leesburg, VA, USA) by a camera mounted from the ceiling above the arena.

3.3 MAGNETIC RESONANCE IMAGING

On PD 28, piglets were transported to the Biomedical Imaging Center at the Beckman Institute and anesthetized using a telazol:ketamine:xylazine solution (100/50/50 mg/kg/BW; Fort Dodge Animal Health, Overland Park, KS, USA). The anesthetic combination was administered intramuscularly at 0.022 ml/kg BW and anesthesia was maintained throughout the scan by inhalation of isoflurane (98% oxygen/2% isoflurane; Henry Schein, Dublin, OH, USA). Once fully anesthetized, piglets were restrained to prevent motion artifacts and placed in the MRI scanner. Vital signs were monitored and recorded every 5 minutes throughout the scanning procedure using an MRI compatible pulse oximeter.

All MRI was conducted using a Siemens MAGNETOM Trio 3T imager and a 32-channel head coil (Siemens, Erlangen, Germany). Anatomic images were acquired using a 3D T1-weighted, magnetization-prepared rapid gradient-echo (T1 MPRAGE) sequence with the following parameters: repetition time = 1,900 ms, echo time = 2.49 ms, inversion time = 900ms, flip angle = 9°, matrix = 256x256, 224 slices with slice thickness = 0.7 mm. The final voxel size was 0.7 mm isotropic across the entire head from the tip of the snout to the cervical/thoracic spinal cord junction, as previously described (74). All methods used for voxel-based

morphometry (VBM), brain region volume estimation analysis, diffusion tensor imaging (DTI), and brain metabolites were performed as previously described (27).

3.4 RNA-SEQUENCING ANALYSIS

3.4.1 RNA Extraction, cDNA synthesis, and RNA-Sequencing Library Preparation

An independent cohort of neonatal piglets (n = 24) was used to analyze the effects of SGA and 2′FL on gene expression patterns in the hippocampal transcriptome. Piglets were treated the same as those used for cognitive testing and MRI, but on PD14 piglets were euthanized and right hippocampi were dissected, snap frozen, and stored at -80°C until RNA could be extracted. Tissue (~50mg) was thawed and homogenized in 1 mL TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA), followed by centrifugation for 10 minutes at 12,000xg at 4°C. The supernatant was then moved to fresh tubes. Chloroform (0.2 mL) was added and vortexed for 10 seconds, incubated at room temperature for 5 minutes, and then centrifuged at 10,000xg for 18 minutes at 4°C. The clear supernatant was then moved to new tubes before 0.4 mL of 200 proof ethanol was added and the samples were vortexed. After, the samples were loaded into RNeasy Mini Kit columns (cat. No. 74104, Qiagen, Germantown, MD, USA) following the manufacturer's protocols beginning at Part 1 step 3. An RNase-free DNase set (cat. No. 79254, Qiagen, Germantown, MD, USA) was used for on-column DNase digestion to remove genomic DNA following the manufacturer's protocol. A NanoDrop Spectrophotometer (NanoDrop Technologies, Inc.) was used to determine RNA concentrations.

Samples were analyzed by the Carver High-Throughput DNA Sequencing and Genotyping Unit (HTS lab, University of Illinois, Urbana, IL, USA) using AATI Fragment Analyzer (Advanced Analytical Technologies, Inc.) to determine RNA integrity and the presence/absence of genomic DNA. Only RNA samples with RNA integrity number (RIN)

greater than 7 were used for sequencing. Ribosomal RNA was removed using a Ribozero Kit (Illumina Inc., San Diego, CA, USA) and TruSeq Stranded RNA-seq libraries were created using the TruSeq Stranded RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) following standard protocols on the HiSeq 4000 in four lanes with single-reads 100nt in length. The pool produced over 1.5 billion reads with average quality scores above 30 from start to finish. The libraries were quantified by qPCR and sequenced on four lanes for 101 cycles from one end of the fragments on a HiSeq 4000 using a HiSeq 4000 sequencing kit version 1. Fastq files were generated and demultiplexed with bcl2fastq v2.17.1.14 Conversion Software (Illumina Inc., San Diego, CA, USA).

3.4.2 Obtaining Gene Counts for RNA-Sequencing Analysis

The 24 libraries were transferred from the HTS lab to the High-Performance Biological Computing Lab (HPCBio, University of Illinois, Urbana, IL, USA) to obtain gene counts. Data were transferred to the Biocluster using Globus and then decompressed. The *Sus scrofa* 10.2 reference genome files were obtained from NCBI annotation release 105. The annotation file was then converted to GTF format for compatibility with STAR (version 2.5.2a), an alignment software. A quality check was performed on the FASTQ files using FASTQC (version 0.11.5) software. The reads had already been adapter trimmed at the HTS lab. Results were collated using MultiQC (version 0.7), and the quality of reads was excellent. Trimmomatic (version 0.33) was used to trim any residual adapter content and low quality bases. Very few reads were lost due to trimming. A quality check was performed on the trimmed FASTQ files using FASTQC (version 0.11.5) software. These were run in the same way as the raw reads. The results were nearly identical to the raw reads, since relatively few reads were trimmed. Alignments were ran

against the *Sus scrofa* genome using STAR software, and approximately 80% of reads aligned. All gene counts were generated using featureCounts in the subread (version 1.5.0) package. Only uniquely aligned reads are considered in featureCounts, and results were collated with MultiQC (version 0.7). Approximately 20% of aligned reads were assigned to a gene. This percentage is quite low and may be due to the quality of the available *Sus scrofa* genome annotation file. It is likely not a very complete annotation file. This is evidenced by the fact that many reads (~ 50%) were assigned to "no features", which means they aligned to the genome but did not align to a known gene

3.4.3 RNA-sequencing Read Fates

All analyses hereafter were done in R (75; v 3.3.2). Only 20.2% to 22.7% of the total reads ended up aligning uniquely within genes, while 56.5% to 59.7% did align uniquely, but not within exonic regions of known genes. Further investigation showed this was not due to DNA contamination or widespread missing gene annotations for *Sus scrofa*. One sample was checked and found to have a further 25% of reads aligning within intronic regions of known genes. Immature, pre-spliced RNAs are abundant in human fetal brain tissue (76), so it is likely these neonatal pig brain samples also have a high abundance of immature RNAs. These immature RNAs would have been sequenced because ribosomal depletion was used instead of polyA selection when making sequencing libraries. Although only ~21.5% of reads aligned uniquely within genes, this resulted in ~13.63 million reads per sample. There was no correlation between sequencing depth and number of genes with at least one read (Pearson correlation coefficient = 0.025), thus, the sequencing depth was sufficient. The numbers of reads per gene were normalized using TMM normalization (77) in the edgeR package (78).

3.4.4 Filtering no/low Expressed Genes

NCBI's *Sus scrofa* 10.2 genome has a total of 38,878 genes in annotation release 105. Often a large percentage of genes are not expressed in the sequenced samples and can be removed to reduce the penalty for testing large numbers of genes. However, what number of reads constitutes real expression is unknown because sequencing errors can lead to false alignments. A standard metric is to consider ~10 read counts per sample, adjusted for sequencing depth, in one or more samples as the threshold. Therefore, a requirement of 1 count per million (after TMM normalization) in at least 3 samples was established to keep a gene. 18,968 genes did not meet this threshold and were filtered out, leaving 19,910 genes to be analyzed for differential expression.

3.4.5 Overall Clustering of Samples and Estimation of Surrogate Variables

Samples were clustered after normalization to find outliers, overall treatment differences, or batch effects. Surrogate variables analysis (79, 80) was used to remove artifacts from the dataset by estimating surrogate variables (SV) corresponding to covariance structures found that were not due to our variables of interest (i.e. size and diet) or the batch effect of sex. Five SV were found, the first two of which corresponded not only to cohort, but to differences between pairs within cohorts, SV3 corrects mainly for difference in a single sample, while SV4 and SV5 appear to correct for additional individual sample and pair effects. The SV can be added to the statistical model to adjust for their effects when calculating differential expression, and can also be removed from individual expression values for visualization purposes.

3.4.6 Gene-level Statistical Analysis and Functional Annotation

Testing for differential expression was done using the limma package's (81) empirical Bayes method (82) for RNASeq data known as the "voom" method, which transforms the count data appropriately for linear modeling using log2[(count + 0.5) / (library size x TMM norm factor + 1) / 1e6))] and also estimates observational (gene) -level weights (83), which adjust for the calculated mean-variance relationship. The seven different contrasts were pulled from the model: the main effects of size and diet, the interaction term and the four logical pairwise comparisons. Multiple hypothesis testing adjustment using the False Discovery Rate (FDR) method (84) was done globally across all seven contrasts together, so that the same raw p-value ended up with the same FDR p-value in all contrasts.

Function annotation information for each gene was pulled from two sources. Bioconductor's (85) org.Ss.eg.db package (86) was used to get Gene Ontology's Biological Process, Cellular Component and Molecular Function terms for *Sus scrofa* genes. Updated KEGG pathways, matched based on Entrez Gene IDs, were pulled directly from KEGG using the KEGGREST package (87).

3.5 BRAIN WEIGHT

After MRI was complete, anesthesia was maintained with 0.5 mL telazol:ketamine:xylazine solution and piglets were euthanized by intracardiac injection of 1 mL sodium pentobarbital (Fatal Plus, 72mg/kg body weight; Vortech Pharmaceuticals, Dearborn, MI, USA). Whole brain tissue, excluding optic nerves, was collected and weighed.

3.6 STATISTICAL ANALYSIS

3.6.1 Weight gain, brain weight, and cognitive testing

Data analysis for weight gain and cognitive testing were conducted using the MIXED procedure of the SAS 9.4 software (SAS Institute, Cary, NC, USA) as a 3-way (size x diet x day) repeated measures ANOVA. Brain weight was analyzed as a 2-way (size x diet) ANOVA using GraphPad Prism 7. Measures that were not significant (i.e. sex, litter, cohort) were removed from the model for all analyses. Significant difference was declared at $p<0.05$, tendency at $p<0.10$. Data are presented as average ± SEM.

3.6.2 Voxel-Based Morphometry

The statistical parametric mapping (SPM) toolbox was used for statistical analysis. A 2-way ANOVA with main effects of size (AGA vs. SGA) and diet (2′FL vs. CON) was performed, no covariates were used, and an ANCOVA was used for global normalization. Pseudo-T statistical maps were generated showing areas where there was a difference (i.e. reduction) in grey or white matter for all comparisons using an uncorrected $p<0.001$. A threshold of at least 20 edge-connected voxels (clusters) was used.

3.6.3 Brain Region Volume Estimation, DTI, and MR-Spectroscopy

Data analysis was conducted as a 2-way (size x diet) ANOVA using GraphPad Prism 7. For individual brain region volume estimation, brain volumes were expressed as absolute (mm^3) or relative (i.e. as a proportion of total brain volume for each piglet) units. No covariates were used. Significance was accepted at $p<0.05$.

CHAPTER FOUR

RESULTS

4.1 BRAIN AND BODY WEIGHTS

AGA piglets were born with an average body weight of 1.45±0.12 kg and SGA piglets were born with an average body weight of 0.82±0.10 kg ($p<0.05$). The average body weights of AGA and SGA piglets were significantly different ($p<0.05$) throughout the study, with no effect of diet (**Figure 1**). At PD 28, AGA piglets had larger brain weights than SGA piglets (48.96 g and 44.85 g, respectively; $p=0.003$). However, the brain to body weight ratio was greater in SGA piglets compared to AGA piglets ($p<0.0001$) suggesting a 'brain-sparing' effect during IUGR in SGA piglets (**Figure 2**). There was no effect of diet on brain or brain to BW ratio of piglets.

4.2 COGNITIVE TESTING

During the acquisition phase of testing, overall piglet performance in the spatial T-maze task improved over time ($p<0.0001$) and SGA piglets made fewer incorrect choices than AGA piglets ($p < 0.05$; **Figure 3**). The size x day interaction was not significant ($p>0.05$). There was a trend for significance of diet ($p=0.051$) such that control piglets made fewer incorrect choices than 2′FL, but the diet x day interaction was not significant ($p=0.192$).

There were no significant interactions or main effects observed during the reversal phase of testing. All piglets improved over time ($p<0.0001$) and reached criterion on day 4.

4.3 MAGNETIC RESONANCE IMAGING

4.3.1 Brain Volume Estimation

Piglet brain volume was affected by birth weight, but not dietary intervention. A main effect of size was observed for total brain volume, where SGA piglets had smaller ($p<0.001$) total brain volumes than their AGA counterparts (**Figure 4**). When absolute brain regions of interest (ROI) values were evaluated, a main effect of size was observed for 12 of 22 ROI, where SGA piglets had smaller ($p<0.05$) volumes as compared to AGA piglets. There was no effect of 2′FL on total brain volume or the 22 ROI evaluated (**Table 1**).

Due to different overall brain volumes of AGA and SGA piglets, relative brain volumes were compared (**Table 2**). Again, no main effect of 2′FL was observed for any ROI. Yet, a main effect of size was observed such that SGA piglets had smaller relative volumes of the hypothalamus ($p=0.034$), internal capsule ($p=0.002$), lateral ventricle ($p=0.030$), midbrain ($p=0.001$), pons ($p<0.001$), putamen ($p<0.001$), thalamus ($p=0.011$), and white matter ($p=0.045$) than AGA piglets.

4.3.2 Voxel-Based Morphometry

Volumetric differences were revealed in absolute grey matter (GM), but not absolute white matter (WM), between treatment groups using voxel-based morphometry (**Table 3**). A comparison of grey matter concentration in which AGA piglets had higher ($p<0.001$) regional peak intensities compared to SGA piglets (AGA > SGA) displayed differences in the left and right cortices, thalamus, cerebellum, left hippocampus, and midbrain. Analyzing regional clusters revealed a main effect of diet in the right cortex, such that GM in CON piglets was more

concentrated than 2′FL piglets ($p<0.001$) as defined by significant peak intensities. This voxel wise comparison displayed no significant interactions or main effects in WM concentration.

4.3.3 Single-Voxel Spectroscopy

Total metabolite concentrations of N-acetylaspartate + N-acetylaspartylglutamate (NAA + NAAG), glutamate + glutamine (Glu + Gln), glycerophosphocholine + phosphocholine (GPC + PCh), creatine + phosphocreatine (Cr + PCr), myo-inositol, and lactate were measured in the hippocampus of AGA and SGA piglets fed control and 2′FL (**Table 5**). Data were excluded from the analysis if the standard deviation percentage exceeded 20%. Myo-inositol and lactate did not meet criteria for analysis and were not included. There were no significant interactions or main effects for any of the metabolites measured.

4.3.4 Diffusion Tensor Imaging

Assessment of cortical white matter, corpus callosum, caudate, internal capsule, thalamus, and both hippocampi was performed using diffusion tensor imaging (DTI; **Table 4**). Analysis of DTI data revealed a main effect of size such that SGA piglets had a significant decrease in fractional anisotropy (FA) within caudate ($p=0.006$), internal capsule ($p=0.037$), right hemisphere white matter ($p=0.002$), total white matter ($p=0.002$), and whole brain ($p=0.002$). No brain regions exhibited significant differences in FA values due to 2′FL supplementation.

Additionally, there were differences observed between SGA and AGA piglets in the caudate in terms of mean diffusivity ($p=0.015$), radial diffusivity ($p=0.011$), and axial diffusivity ($p=0.024$). No other brain regions exhibited significant interactions or main effects for these measures.

4.4 RNA-SEQUENCING ANALYSIS

Overall, 529 differentially expressed genes (DEGs) were revealed in hippocampal tissue obtained from piglets on PD14 ($p<0.006$; **Figure 5**). A total of 115 DEGs were identified in response to size, of which 62 were up-regulated and 53 were down-regulated in SGA piglets compared to AGA piglets. A main effect of diet revealed 252 DEGs, of which 144 up-regulated and 108 down-regulated genes in 2′FL piglets compared to CON. There was an interaction among 162 genes where 73 were up-regulated and 89 were down-regulated. The Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.8; 88, 89) tool was used for functional enrichment analysis.

DAVID utilizes Gene Ontology (GO) classifications to understand the trends in transcriptional response between each main effect and the interaction term (90). This system characterizes genes using defined categories of molecular function (MF), biological process (BP), and cellular component (CC). Only annotation clusters with enrichment scores >1.3 were included in the analysis. The GO categories for up-regulated genes between the SGA and AGA groups were associated with chromatin silencing, DNA binding, nuclear chromatin, and the nucleosome. Interestingly, down-regulated genes between SGA and AGA groups were associated with KEGG pathways for measles, influenza A, hepatitis C, and herpes simplex infection. Moreover, genes that were up-regulated in response to 2′FL were connected to integral components of membranes and transmembrane helix, while down-regulated genes were clustered based on ion channel and transport, zinc ion binding, zinc-finger, and metal-binding. Finally, in the size by diet interaction, there were no clusters discovered as a result of up-regulated gene expression, however, down-regulated genes were associated with transmembrane helix, membranes, and integral components of membrane.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

Intrauterine growth restriction often leads to small for gestational neonates, increasing risk of cognitive impairments and learning deficits that persist into adulthood. Rapid growth and expansion of the brain occurs during the last trimester and the first year of life leaving it vulnerable to insults that may be mitigated with proper early-life nutrition. The benefits of breastfeeding have been well established and many studies have validated the positive impact of human milk on the cognitive development of neonates. While exclusive breastfeeding rates continue to rise, numerous mothers rely on infant formula. While formula can promote efficient growth, development, and nutrient balance of the infant (44), there are functional ingredients that it lacks leading human milk to be far superior. Therefore, it is imperative to develop products that close the gap between the two in order to ensure proper neonatal nutrition.

Due to their abundance in human milk, HMOs have become a major target for improving formula. It has been postulated that HMOs play a key role in brain development due to their high abundance and variety as compared to bovine-milk based formulas (17). Yet, with over 200 identified, it is not economical or practical for infant formula to contain the full repertoire of HMOs. Thus, by identifying the key HMOs in breast milk, it is possible that the brain development of infants fed formula can be more similar to that of the breastfed neonate. Recent studies have demonstrated 2′FL, the most abundant HMO, enhances brain and cognitive development (21-23). To our knowledge, there has been no published data investigating the effect of dietary 2′FL on the brain and cognitive development of SGA infants. Therefore, the present study utilized a neonatal piglet model to determine if orally administered 2′FL could enhance brain and cognitive development in infants born SGA due to IUGR.

Piglet learning and memory was tested using a hippocampal dependent spatial T-maze task. Chocolate milk was present in both reward arms to balance for olfactory cues, and the start arm location was randomized throughout testing. Thus, piglets were forced to rely on extra-maze visual cues to create a spatial map (i.e. allocentric mechanism) instead of a striatum-dependent egocentric mechanism (i.e. turning body left or right) for solving the task (28). Our group has previously reported a learning deficit in SGA piglets using the spatial T-maze task, whereby SGA piglets required several additional days to reach criterion as well as made more errors than AGA piglets (27). Interestingly, in the present study SGA piglets reached criterion faster as well as made fewer errors than their AGA counterparts. However, there are important differences to note between the experimental designs of these studies. In the former study, piglets were supplied milk replacer at 285 ml/kg BW while piglets in our present study were fed at 300 ml/kg. Furthermore, an automated milk delivery system was utilized in our previous study, whereby piglets were fed their daily-allotted milk over 18 meals (once per hour) followed by a 6-h fasting period prior to behavioral testing. The present study supplied 5 meals (once every 3 hours) per day followed by a 10-h fasting period prior to behavior testing. In the prior study, the overall body weights of AGA and SGA piglets were lower than those in the present study, and the SGA piglets experienced compensatory "catch up" growth such that the average weight of SGA and AGA piglets was similar by PD 15 and throughout behavioral testing. In the present study, AGA and SGA final body weights were significantly different, with no effect of 2′FL, throughout the study. Moreover, in the present study, the brain weights were significantly different between SGA and AGA piglets on PD 28, while the brain weights in the prior study were not different. The differences identified between the two studies must be considered when comparing their results.

Performance of SGA piglets in the spatial T-maze task was opposite to what we expected and differs from results in human studies. Yet, Antonides et al. (91) found similar results when evaluating cognitive performance of very low birth weight (VLBW) piglets. Using a holeboard task to test spatial learning and memory, they found that VLBW piglets acquired the task faster and reached a higher performance level than normal birth weight piglets. Taken together, our results could be the outcome of a compensatory mechanism developed in utero to increase piglet survivability. Furthermore, it is possible that SGA piglets are more strongly motivated to obtain a food reward than AGAs. Further research is needed to measure food motivation and cognitive compensatory mechanisms in SGA piglets.

In the present study, 2′FL did not enhance neonatal piglet performance in the spatial T-maze task. Oliveros et al. (22) found similar results utilizing a novel object recognition task for young and adult rats. In this study, there were no differences found between 2′FL and CON groups in young rats, but adult 2′FL rats spent more time exploring novel objects than the CON animals did. It is possible that the 2′FL supports a long-term cognitive effect, and the animals utilized in the present study were too young to observe a performance difference. Moreover, it is possible that the spatial T-maze is not a sensitive enough behavioral task to identify the benefits of 2′FL. Vazquez et al. (21) utilized hippocampal long-term potentiation (LTP) to understand the role of 2′FL on gut-brain communication. LTP is an electrophysiological technique that has been considered a key cellular mechanism underlying learning and memory processes. Better learning skills would be reflected in higher and longer-lasting LTP. To our knowledge, LTP has yet to be utilized in the neonatal piglet model, but it could shed light on the role of 2′FL on the SGA brain.

For SGA human infants, catch-up growth is common and typically associated with decreased mortality within the first few months after birth (92). It has also been connected with a

lowered risk of neurologic and intellectual dysfunction in early adulthood (93). Yet, catch-up growth is not commonly seen in the swine industry. When grown out to slaughter age, low birth weight pigs showed the lowest growth performance and lean percentage compared to normal birth weight pigs (94). This is consistent with our data, such that SGA piglets never experienced catch-up growth and remained significantly smaller than their AGA counterparts throughout the entire course of the study. It's important to note that our piglets were limit fed, which may have prevented them from demonstrating catch-up growth. 2′FL had no effect on piglet growth throughout the study. However, this is consistent with other studies orally administering 2′FL. A 3-week pre-clinical study in farm piglets observed no growth and developmental effects of various concentrations of 2′FL (95). In this study, male and female piglets fed 2′FL showed consistent increase in BW over time when compared to control piglets. This could also explain the lack of brain growth and maturation seen from the MRI analysis as well.

In the present study, the assessment of total brain volume revealed SGA piglets had significantly smaller brains at PD 28. However, the brain to BW ratio of SGA piglets was much larger than that of AGA piglets, suggesting a "brain-sparing" effect. The most common cause of IUGR is placental insufficiency, and this often results with the fetus adapting its circulation to preserve oxygen and nutrient supply to the brain (96). The brain-sparing effect is implemented as a protective mechanism, yet several studies have shown that IUGR subjects with brain-sparing display worse neurodevelopmental and behavioral outcomes when compared to symmetric IUGR and controls (96). Prolonged brain-sparing, such as that seen in the present study, may lead to altered structure and function of the cerebral vasculature.

Absolute volumetric assessment of specific brain regions revealed 12 significantly smaller regions in SGA piglets as compared to AGA piglets, with no effect of 2′FL. Yet, when

these regions were expressed relative to total brain volume, only the midbrain, pons, and thalamus were still affected by SGA status. Interestingly, the hypothalamus, internal capsule, and lateral ventricle of SGA piglets were significantly smaller than AGA piglets when expressed relative to total brain volume. These regions were not significant as a result of the absolute volumetric assessment. Taken together, these results suggest SGA neonates have proportionately smaller subcortical structures, giving reason to believe there is preferential development of these regions as a result of IUGR.

VBM grey matter (GM) outcomes support this idea of preferential development of subcortical regions. AGA piglets exhibit higher GM volume percentages in the cerebellum, thalamus, hippocampus, and midbrain when compared to SGA piglets, suggesting these regions are more mature in AGA neonates. While VBM did not reveal any volumetric WM differences, diffusion tensor imaging (DTI) was utilized to evaluate WM maturation in piglet brains. DTI sequences allow for interpretation of water movement within the brain. As the composition and morphology of the brain changes over time, the movement of water is also expected to change reflecting the development and maturation of WM tracts. WM tract development is the most intense during the perinatal period, but begins during the prenatal period and continues through adulthood (97). SGA neonates have been connected to a reduction in WM integrity and increase in prevalence of psychiatric symptoms in adulthood (98). In the present study, there was reduced fractional anisotropy (FA) in the caudate, internal capsule, whole brain, right hemisphere white matter, and total white matter of SGA piglets. This reduction in FA typically represents damaged or reduced organization of WM bundles, and it is often connected to axon injury or reductions in myelination (98). This suggests that SGA neonates have microstructural deficits in these brain regions that are likely caused by reduced number of axons and myelination.

Aside from FA, there are three other outcome measurements obtained from DTI sequences. Mean diffusivity (MD) measures the total water movement within a specific region, radial diffusivity (RD) measures water movement perpendicular to axonal alignment, and axial diffusivity (AD) is utilized to measure the movement of water parallel to axonal alignment. There was no effect of 2′FL, but SGA piglets had an increase in MD, RD, and AD in the caudate when compared to AGA piglets. This finding is not surprising because AGA piglets had significantly larger brain volumes than SGA piglets and MD typically decreases as brain microstructure increases in size (99). As axons mature, they become wrapped in myelin. This results in decreased RD and AD values as less water is able to move across the axon. Thus, a higher RD or AD would be indicative of less myelination. The caudate nuclei are an essential part of the brain's learning and memory system. However, research has shown a modulatory role of the caudate in the processing of egocentric spatial cues and not allocentric spatial cues (100), as seen in the spatial T-maze task. Therefore, further tests are needed to understand if there are any behavioral modifications associated with the microstructural deficits in the caudate of SGA neonates.

MRS did not reveal any significant interactions or main effects in hippocampal metabolites in the present study. This is consistent with data in our previous study comparing SGA and AGA neonatal piglets (27), and it is important to note that Roelants-van Rijn et al. (101) discovered similar brain metabolite concentrations between SGA and AGA preterm human infants

Due to the limited knowledge of the influence of 2′FL on learning and memory in SGA neonates, we utilized RNA-sequencing technology to identify global differences in hippocampal gene transcription. The gene ontology and pathway analysis suggested that the differentially

expressed genes due to size, most of which were up-regulated in SGA compared to AGA, were associated with chromatin silencing, DNA binding, nuclear chromatin, histone core, and the nucleosome. DNA and histones are the basic components of a chromosome. Recent studies have shown that the regulation of higher-order chromatin structures by DNA methylation and histone modification is critical for genome reprogramming en utero, and for tissue-specific gene expression and global gene silencing (102). If chromatin modification is disrupted, it can lead to the dysregulation of developmental processes and various diseases. Further research is needed to understand the role of epigenetics and IUGR.

Interestingly, down-regulated genes between SGA and AGA groups were associated with KEGG pathways for measles, influenza A, hepatitis C, and herpes simplex infection. However, the same 4 genes were clustered into these annotation categories: DExD/H-box helicase 58 (DDX58), adenosine deaminase (ADAR), interferon regulatory factor 9 (IRF9), and interferon-induced double-stranded RNA-activated protein kinase. DDX58 is involved in the innate immune defense against viruses while ADAR is a protein-coding gene that has shown to increase gene product diversity (103). Further research is needed to elucidate the effect of down-regulating these genes in IUGR neonates.

To our knowledge, there is no published data on the effect of 2′FL on the hippocampal transcriptome. Genes that were up-regulated in response to 2′FL were connected to integral components of membranes and transmembrane helix, while down-regulated genes were clustered based on ion channel and transport, zinc ion binding, zinc-finger, and metal-binding. Taken together, it appears that 2′FL modulates channels and membranes, possibly permitting or reducing the transport of substances across the biological membrane.

In the size by diet interaction, there were no clusters discovered as a result of up-regulated gene expression, however, down-regulated genes were associated with transmembrane helix, membranes, and integral components of membrane. It seems that both 2′FL and SGA modulate the epigenome, but again, further research is necessary to understand pathophysiology of these epigenetic changes.

In conclusion, the present study found that the volumetric and microstructural deficits observed in SGA piglets did not influence spatial learning and memory. While we found no evidence that 2′FL affected brain structure or behavior, it modified the expression of genes associated with membranes, transporters, and ion channels in hippocampal tissue. Further study is needed to assess if this differential gene expression is beneficial to the SGA neonate. Nonetheless, the present study suggests 2′FL can be provided to vulnerable IUGR neonates without adversely affecting growth and brain development. This is important because the most widely reported effect of 2′FL is to enhance immune development.

CHAPTER SIX

FUTURE DIRECTIONS

There are many possible directions to take in order to enhance our understanding of the role of 2′FL on learning and memory in SGA neonates. Since no differences were found using the spatial T-maze task between PD 14 and 24, a later time point could be used to see if there are long-term benefits of consuming 2′FL during the postnatal period. Furthermore, a more sensitive measurement of learning and memory could be utilized instead of the spatial T-maze task. It is possible that the beneficial effects of 2′FL were so subtle that the T-maze didn't reveal them, thus a more sensitive measurement (e.g. LTP) could be utilized to elucidate a role of 2′FL in learning and memory. Moreover, it is possible that SGA piglets developed a compensatory mechanism in utero that programmed them to be more motivated by a food reward. Other behavioral tests, such as novel object recognition, could remove this potentially confounding factor.

While there was no effect of SGA on spatial learning and memory, DTI results indicate that there was less myelination in the caudate nucleus. The caudate has shown a modulatory role in egocentric learning and memory (100), and further research could identify if SGA neonates have deficits in striatum-dependent learning. 2′FL may be used as an intervention to attenuate these deficits if they exist.

Finally, the RNA-sequencing data suggest that there are epigenetic modifications that result from IUGR. Epigenetics involve changing gene expression without changing the underlying DNA sequence. DNA methylation and histone modifications (i.e. acetylation, methylation, phosphorylation) are types of epigenetic mechanisms that alter DNA accessibility and chromatin structure, thus regulating patterns of gene expression (104). Early life adversity,

such as being born SGA, has shown to alter DNA methylation in the brain and peripheral tissues, and often lead to adverse phenotypic changes (105). DNA methylation is potentially reversible and preventable, and further research could identify specific genes to target with epigenetic therapeutic interventions.

FIGURES AND TABLES

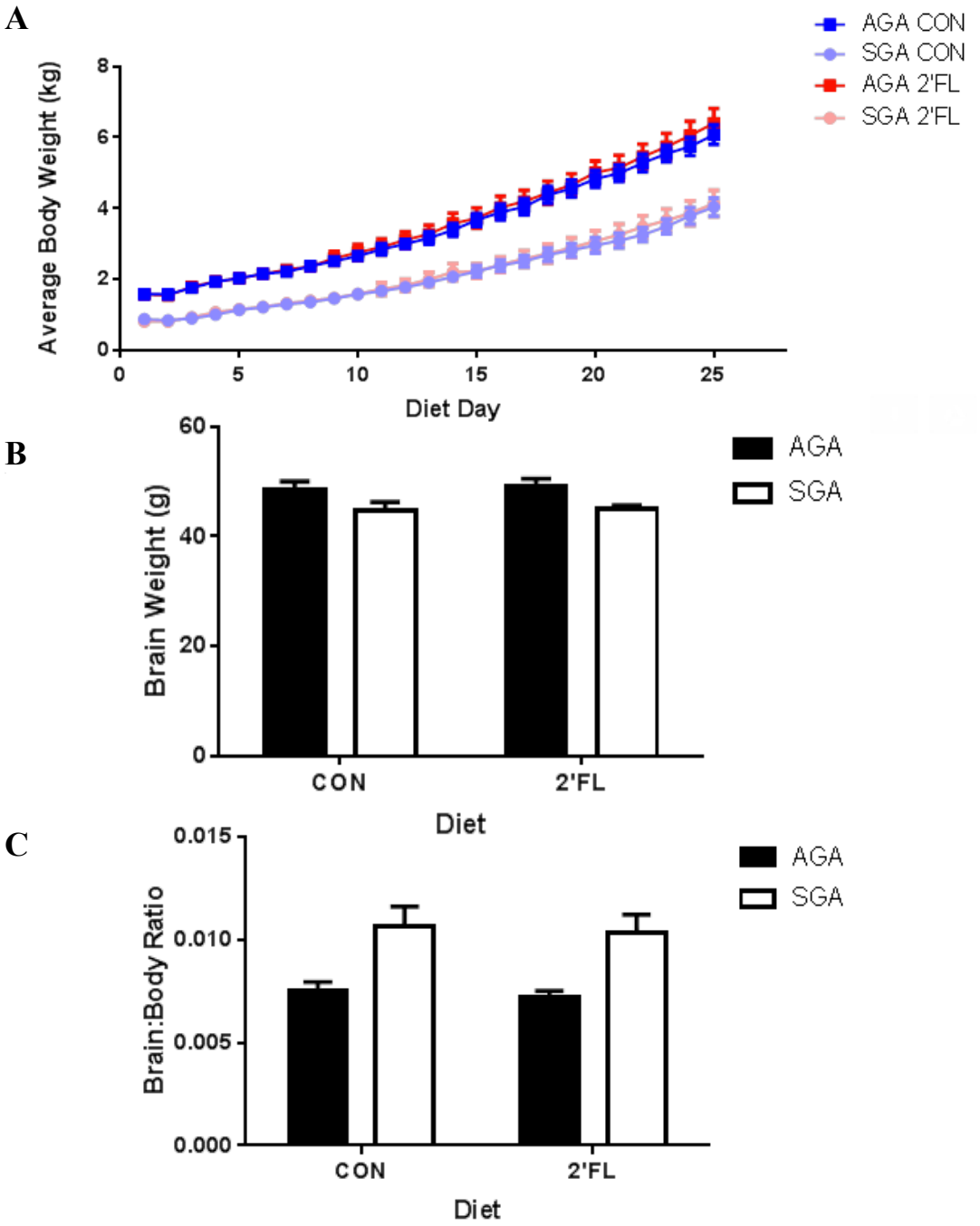

FIGURE 1. Body weight (A) of AGA and SGA piglets increased over time ($p<0.0001$), but body weights of AGA and SGA piglets were significantly different throughout the entire study ($p<0.0001$). Wet brain weight (B) and brain:body weight ratio (C) of AGA and SGA piglets were significantly different ($p<0.0001$, $p<0.01$, and $p<0.0001$, respectively). No significant effect was found from dietary treatment. Data are presented as mean ± SEM, n=9 per group.

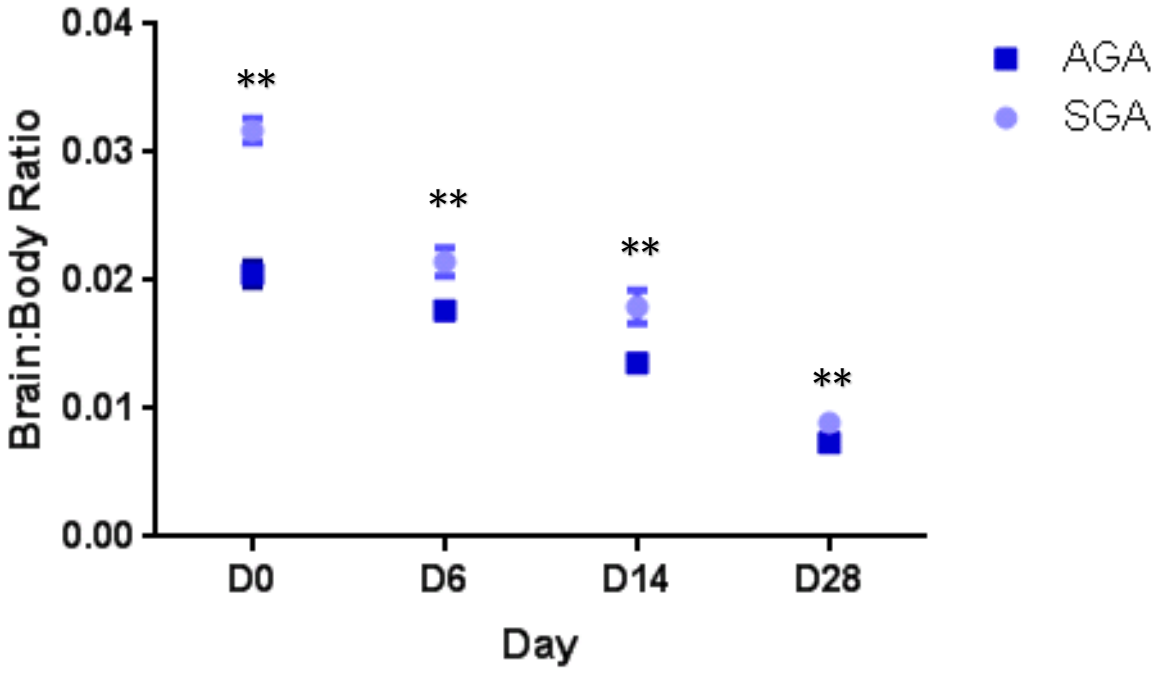

FIGURE 2. The difference between the brain to body weight ratio of AGA and SGA piglets decreases over time, but is still significantly different at PD28 ($p<0.001$). All time-points are different cohorts of piglets. Data are shown as means ± SEM.

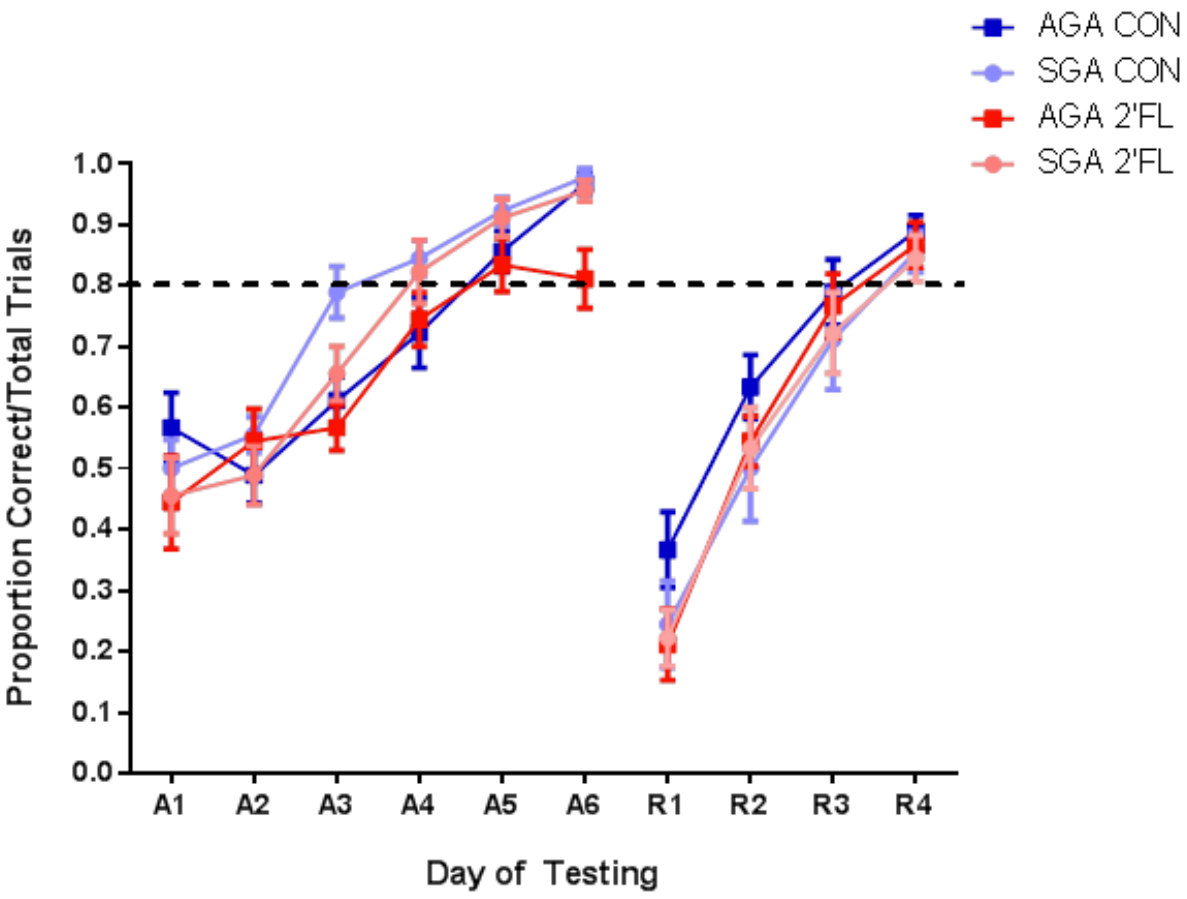

FIGURE 3. Performance of all piglets during acquisition and reversal phases of spatial T-maze task improved over time ($p<0.0001$), but SGA piglets reached criterion faster than AGA piglets ($p<0.05$), with no effect of diet.

Table 1. Effects of SGA and 2′FL on brain region volumes in mm^3 of 4-wk-old piglets[1]

Region of Interest	Treatment				Pooled SEM	P – value[2]		
	AGA CON	SGA CON	AGA 2′FL	SGA 2′FL		Size	Diet	Size x Diet
n	4	7	4	7				
Caudate	527.8	471.3	494.6	485.7	43.7	0.152	0.672	0.290
Cerebellum	6488	5730	6355.4	5755.5	325.8	**<0.001**	0.746	0.633
Cerebrum	51.7	49	47.3	46.6	7.0	0.622	0.347	0.775
Corpus Callosum	378.2	360.7	381.9	325.9	70.7	0.312	0.666	0.592
Fourth Ventricle	54.1	48.3	46.6	50.4	7.3	0.789	0.470	0.201
Grey Matter	39420.4	33698.4	38454.4	33548.3	2783.9	**0.001**	0.693	0.773
Hypothalamus	253.6	237.9	257.1	244.1	15.4	0.078	0.534	0.862
Internal Capsule	1544.1	1486.8	1523.8	1479.1	96.3	0.303	0.774	0.897
Lateral Ventricle	468.1	513.2	443.7	503.1	81.1	0.214	0.675	0.862
Left Cortex	17839.8	15662	17022.7	15603.6	1061.9	**0.003**	0.420	0.484
Left Hippocampus	510.9	447	492.4	453.8	35.3	**0.010**	0.743	0.483
Medulla	1919.2	1742.4	1865	1765.9	114.6	**0.027**	0.792	0.506
Midbrain	1942.9	1813.6	1886.9	1823.5	72.3	**0.016**	0.531	0.374
Olfactory Bulb	2593.9	2280.9	2471.8	2222.6	213.4	**0.017**	0.409	0.768
Pons	1134.4	1055.6	1074.4	1046	50.6	**0.048**	0.186	0.334
Putamen	405.4	393.3	393.8	393.5	25.4	0.631	0.658	0.645
Right Cortex	18374.7	16209.3	17520.9	16084.1	1019.5	**0.002**	0.350	0.484
Right Hippocampus	515.7	459.2	505.1	463.2	32.5	**0.007**	0.841	0.657
Thalamus	1722.1	1573.8	1677.1	1590.9	81.6	**0.010**	0.737	0.456
Third Ventricle	49.1	46.6	46.5	43.8	6.2	0.408	0.387	0.983
White Matter	16248.5	15360.8	16225.8	15445.4	1184.3	0.176	0.959	0.929
Whole Brain	68656	59919.2	65967.5	60239.9	3240.5	**<0.001**	0.474	0.365

[1]Values are means of 4-7 replicate pigs with MRI data collected at 27-29 d of age. Volumes are in units of mm^3.

[2]Size, main effect of birth weight (i.e. AGA vs. SGA); Diet, main effect of dietary intervention (i.e. 2′FL vs. CON); Size x Diet, interaction effect of birth weight and dietary intervention.

Abbreviations: AGA, appropriate for gestation age; SGA, small for gestational age; CON, control; 2′FL, 2′-fucosyllactose

Table 2. Effects of SGA and 2′FL on relative brain region volumes (% of whole brain) of 4-wk-old piglets[1]

Region of Interest	Treatment				Pooled SEM	P – value[2]		
	AGA CON	SGA CON	AGA 2′FL	SGA 2′FL		Size	Diet	Size x Diet
n	4	7	4	7				
Caudate	0.77	0.79	0.75	0.81	0.05	0.141	0.936	0.408
Cerebellum	9.45	9.56	9.63	9.55	0.33	0.973	0.626	0.576
Cerebrum	0.08	0.08	0.07	0.08	0.01	0.268	0.446	0.978
Corpus Callosum	0.55	0.60	0.58	0.54	0.11	0.946	0.734	0.415
Fourth Ventricle	0.08	0.08	0.07	0.08	0.01	0.279	0.649	0.392
Grey Matter	57.42	56.24	58.29	55.69	2.80	0.177	0.832	0.648
Hypothalamus	0.37	0.40	0.39	0.41	0.02	**0.034**	0.139	0.573
Internal Capsule	2.25	2.48	2.31	2.46	0.11	**0.002**	0.711	0.405
Lateral Ventricle	0.68	0.86	0.67	0.84	0.14	**0.030**	0.755	0.930
Left Cortex	25.98	26.14	25.80	25.90	0.67	0.763	0.622	0.960
Left Hippocampus	0.74	0.75	0.75	0.75	0.03	0.837	0.739	0.814
Medulla	2.80	2.91	2.83	2.93	0.11	0.066	0.597	0.905
Midbrain	2.83	3.03	2.86	3.03	0.09	**0.001**	0.782	0.735
Olfactory Bulb	3.78	3.81	3.75	3.69	0.03	0.886	0.633	0.774
Pons	1.65	1.76	1.63	1.74	0.05	**<0.001**	0.330	0.943
Putamen	0.59	0.66	0.60	0.65	0.02	**<0.001**	0.851	0.687
Right Cortex	26.76	27.05	26.56	26.70	0.54	0.473	0.366	0.811
Right Hippocampus	0.75	0.77	0.77	0.77	0.03	0.534	0.491	0.745
Thalamus	2.51	2.63	2.54	2.64	0.08	**0.011**	0.512	0.825
Third Ventricle	0.07	0.08	0.07	0.07	0.01	0.413	0.515	0.753
White Matter	23.67	25.64	24.60	25.64	1.39	**0.045**	0.494	0.487

[1]Values are means of 4-7 replicate pigs with MRI data collected at 27-29 d of age. Relative volumes of regions of interest were obtained by determining the percent of whole brain volume for individual pigs prior to statistical analysis.

[2]Size, main effect of birth weight (i.e. AGA vs. SGA); Diet, main effect of dietary intervention (i.e. 2′FL vs. CON); Size x Diet, interaction effect of birth weight and dietary intervention.

Abbreviations: AGA, appropriate for gestation age; SGA, small for gestational age; CON, control; 2′FL, 2′-fucosyllactose

Table 3. Effects of SGA and 2′FL on grey matter percentage volumes as determined by voxel-based morphometric analysis

	Region of Interest	Number of Voxels[1]	Peak-level P-value[2]	Local maxima coordinates x	y	z
Main Effect of Size (AGA > SGA)	Cerebellum	1661	<0.001	11.9	-18.2	0.7
	Right Cortex	2849	<0.001	2.1	-12.6	14.7
	Thalamus	244	<0.001	3.5	8.4	2.1
	Cerebellum	123	<0.001	9.8	-14.0	-11.2
	Left Cortex	96	<0.001	-5.6	11.2	14.7
	Left Cortex	228	<0.001	-5.6	27.3	1.4
	Right Cortex	308	<0.001	4.2	4.2	16.1
	Cerebellum	33	<0.001	-10.5	-14.7	-11.2
	Right Cortex	57	<0.001	0.0	23.8	-2.1
	Left Hippocampus	42	<0.001	11.9	19.6	10.5
	Right Cortex	77	<0.001	7.7	21.7	-6.3
	Left Cortex	238	<0.001	-14.0	4.9	12.6
	Midbrain	51	<0.001	7.0	-4.9	-7.7
Main Effect of Diet (CON > 2′FL)	Right Cortex	32	<0.001	14.7	11.2	4.2
Size x Diet	No significant findings					

[1]Clusters > 20 were included in analysis with threshold set at p<0.001
[2]uncorrected P-values presented

Table 4. Effects of SGA and 2′FL on white matter maturation as determined by diffusion tensor imaging in 4-wk-old piglets[1]

	Treatment					P – Value[2]		
Region of Interest	AGA CON	SGA CON	AGA 2′FL	SGA 2′FL	Pooled SEM	Size	Diet	Size x Diet
Fractional Anisotropy, FA Units								
Corpus Callosum	0.269	0.263	0.282	0.266	0.014	0.141	0.279	0.451
Caudate	0.330	0.291	0.307	0.295	0.016	**0.006**	0.255	0.112
Internal Capsule	0.417	0.403	0.410	0.397	0.012	**0.037**	0.327	0.944
Thalamus	0.300	0.320	0.316	0.326	0.018	0.117	0.239	0.552
Left Hippocampus	0.293	0.299	0.296	0.302	0.017	0.524	0.743	0.989
Right Hippocampus	0.278	0.287	0.289	0.291	0.015	0.496	0.338	0.658
Cerebellum	0.165	0.167	0.166	0.169	0.011	0.713	0.768	0.867
Left White Matter	0.315	0.308	0.310	0.310	0.004	0.091	0.414	0.078
Right White Matter	0.312	0.307	0.316	0.306	0.004	**0.002**	0.616	0.188
Total White Matter	0.313	0.307	0.312	0.307	0.003	**0.002**	0.769	0.736
Whole Brain	0.316	0.309	0.314	0.310	0.003	**0.002**	0.692	0.248
Radial Diffusivity, x 10^-3 mm^2 / sec								
Corpus Callosum	1.124	1.172	1.076	1.046	1.455E-04	0.901	0.254	0.609
Caudate	0.785	0.906	0.809	0.923	8.153E-05	**0.011**	0.633	0.926
Internal Capsule	0.650	0.660	0.653	0.655	2.041E-05	0.562	0.883	0.701
Thalamus	0.743	0.750	0.754	0.758	2.000E-05	0.589	0.371	0.872
Left Hippocampus	0.997	1.037	1.033	1.018	1.131E-04	0.825	0.882	0.640
Right Hippocampus	0.993	0.978	1.029	0.982	7.401E-05	0.419	0.595	0.677
Cerebellum	1.064	1.003	1.009	1.039	5.429E-05	0.587	0.721	0.119
Left White Matter	0.819	0.831	0.835	0.824	1.970E-05	0.924	0.691	0.267
Right White Matter	0.804	0.817	0.820	0.825	1.961E-05	0.375	0.250	0.700
Total White Matter	0.811	0.825	0.827	0.823	1.872E-05	0.581	0.472	0.383
Whole Brain	0.822	0.836	0.837	0.837	2.069E-05	0.530	0.456	0.530
Axial Diffusivity, x 10^-3 mm^2 / sec								
Corpus Callosum	1.695	1.744	1.661	1.575	2.070E-04	0.868	0.346	0.528
Caudate	1.311	1.413	1.296	1.453	1.030E-04	**0.024**	0.807	0.610
Internal Capsule	1.277	1.266	1.273	1.242	2.122E-05	0.065	0.211	0.343
Thalamus	1.170	1.236	1.229	1.262	5.298E-05	0.080	0.135	0.547
Left Hippocampus	1.573	1.642	1.632	1.612	1.495E-04	0.752	0.852	0.570
Right Hippocampus	1.528	1.528	1.629	1.551	9.491E-05	0.432	0.213	0.434
Cerebellum	1.352	1.283	1.297	1.326	6.330E-05	0.538	0.850	0.145
Left White Matter	1.329	1.335	1.346	1.324	2.353E-05	0.513	0.785	0.258
Right White Matter	1.303	1.313	1.334	1.323	2.785E-05	0.968	0.170	0.461
Total White Matter	1.315	1.326	1.339	1.321	2.391E-05	0.778	0.453	0.258
Whole Brain	1.339	1.348	1.357	1.350	2.772E-05	0.964	0.469	0.582
Mean Diffusivity, x 10^-3 mm^2 / sec								
Corpus Callosum	1.314	1.363	1.270	1.223	1.655E-04	0.995	0.288	0.574
Caudate	0.961	1.075	0.972	1.099	8.806E-05	**0.015**	0.699	0.884
Internal Capsule	0.859	0.862	0.860	0.850	1.928E-05	0.762	0.571	0.538
Thalamus	0.885	0.912	0.912	0.926	2.844E-05	0.178	0.178	0.660
Left Hippocampus	1.189	1.239	1.232	1.216	1.245E-04	0.793	0.870	0.611
Right Hippocampus	1.172	1.161	1.229	1.172	8.001E-05	0.416	0.410	0.576
Cerebellum	1.160	1.096	1.105	1.134	5.714E-05	0.566	0.770	0.128
Left White Matter	0.989	9.990	1.005	0.991	2.083E-05	0.863	0.720	0.248
Right White Matter	0.970	0.982	0.991	0.991	2.213E-05	0.618	0.208	0.598
Total White Matter	0.979	0.992	0.997	0.989	2.024E-05	0.814	0.463	0.319
Whole Brain	0.995	1.007	1.011	1.008	2.294E-05	0.684	0.470	0.547

[1]Values are means of 4-7 replicate pigs with MRI data collected at 27-29 d of age.

[2]Size, main effect of birth weight (i.e. AGA vs. SGA); Diet, main effect of dietary intervention (i.e. 2′FL vs. CON); Size x Diet, interaction effect of birth weight and dietary intervention

Table 5. Effects of birth weight and 2′FL on hippocampal metabolism as determined by single-voxel spectroscopy in 4-wk-old piglets[1]

	Treatment					P – Value[2]		
Metabolite	AGA CON	SGA CON	AGA 2′FL	SGA 2′FL	Pooled SEM	Size	Diet	Size x Diet
n	9	9	8	9				
NAA + NAAG	5.014	5.739	5.472	5.581	3.778	0.279	0.695	0.423
GPC + PCh	1.287	1.443	1.301	1.494	1.011	0.146	0.784	0.877
Cr + PCr	4.222	4.770	4.602	4.575	3.097	0.271	0.693	0.225
n	8	8	6	9				
Glu + Gln	7.933	8.424	8.725	8.558	6.222	0.770	0.407	0.555

[1]Values are means of 6-9 replicate pigs with MRI data collected at 27-29 d of age

[2]Size, main effect of birth weight (i.e. AGA vs. SGA); Diet, main effect of dietary intervention (i.e. 2′FL vs. CON); Size x Diet, interaction effect of birth weight and dietary intervention.

Abbreviations: AGA, appropriate for gestational age; SGA, small for gestational age; 2′FL, 2′-fucosyllactose; NAA + NAAG, N-acetylaspartate + N-acetylaspartylglutamate; GPC + PCh, glycerophosphocholine + phosphocholine; Cr + PCr, creatine + phosphocreatine; Glu + Gln, glutamate + glutamine

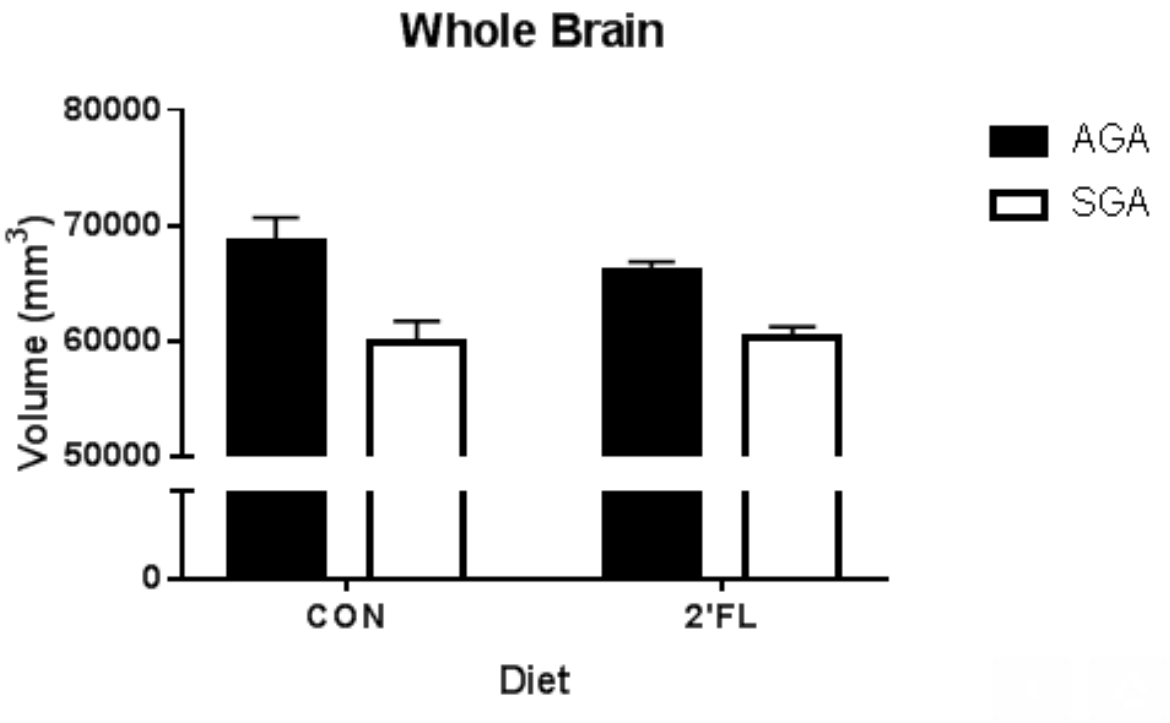

Figure 4. Brain volume measured by MRI did not reveal a significant effect of dietary treatment for the whole brain, but a main effect of size was observed such that SGA brain volume was smaller than AGA ($p<0.001$). Data are presented as means ± SEM.

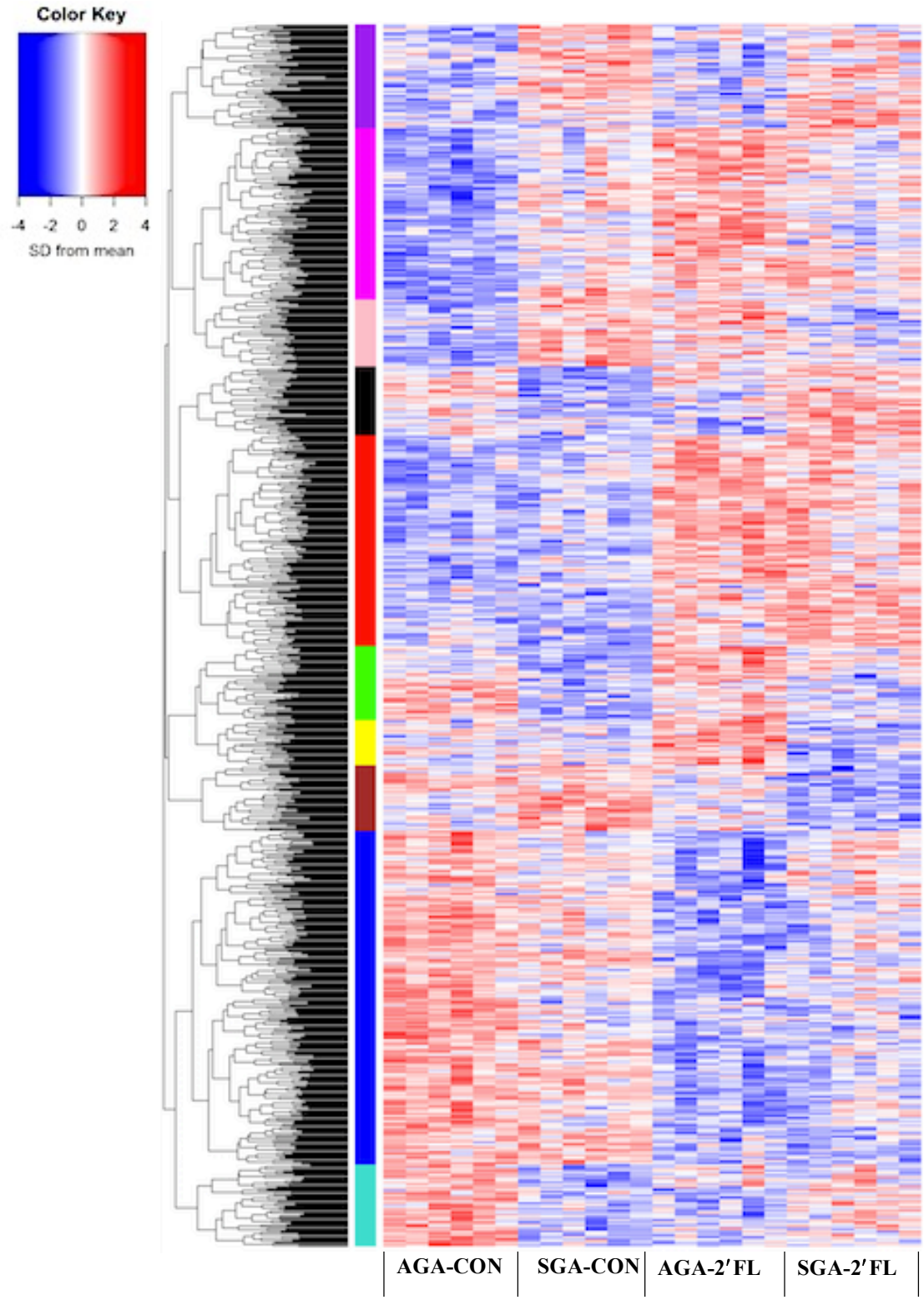

Figure 5. Visualization of relative expression of the 529 DEGs in each piglet (blue=down-regulation; red=up-regulation). Each row represents a gene and its relative expression. Piglets were clustered on overall expression patterns.

References

1) Cosmi E, Fanelli T, Visentin S, Trevisanuto D, Zanardo V. Consequences in infants that were intrauterine growth restricted. Journal of Pregnancy. 2011;2011:1-6.

2) Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. Journal of Animal Science. 2006;84:2316-2337.

3) De Onis M, Blössner M, Villar J. Levels and patterns of intrauterine growth retardation in developing countries. European Journal of Clinical Nutrition. 1998;52:S5.

4) Wollmann HA. Intrauterine growth restriction: definition and etiology. Hormone Research. 1998;49:1-6.

5) Tolsa CB, Zimine S, Warfield SK, Freschi M, Rossignol AS, Lazeyras F, Hanquinet S, Pfizenmaier M, Huppi PS. Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. Pediatric Research. 2004;56:132-138.

6) Geva R, Eshel R, Leitner Y, Valevski AF, Harel S. Neuropsychological outcome of children with intrauterine growth restriction: a 9-year prospective study. Pediatrics. 2006;118:91-100.

7) Courchesne E, Chisum HJ, Townsend J, Cowles A, Covington J, Egaas B, Harwood M, Hind S, Press GA. Normal brain development and aging: quantitative analysis at in vivo MR imaging in healthy volunteers. Radiology. 2000;216:672-682.

8) Gogtay N, Nugent TF III, Herman DH, Ordonez A, Greenstein D, Hayashi KM, Clasen L, Toga AW, Giedd JN, Rapoport JL, et al. Dynamic mapping of normal human hippocampal development. Hippocampus. 2006;16:664-672.

9) McEwen BS. Stress and hippocampal plasticity. Annual Review of Neuroscience. 1999;22:105-122.

10) Strauss RS. Adult functional outcome of those born small for gestational age. The Journal of the American Medical Association. 2000; 283:625-632.

11) Walker A. Breast milk as the gold standard for protective nutrients. The Journal of Pediatrics. 2010;156:S3-7.

12) Hanson LA. Breastfeeding provides passive and likely long-lasting active immunity. Annals of Allergy, Asthma, and Immunology. 1998;81:523-534.

13) Chandra RK. Prospective studies of the effect of breast feeding on incidence of infection and allergy. Acta Paediatrica. 1979;68:691-694.

14) Kramer MS, Aboud F, Mironova E, et al. Breastfeeding and child cognitive development. Archives General Psychiatry. 2008;65:578-584.

15) Fergusson DM, Beautrais AL, Silva PA. Breast-feeding and cognitive development in the first seven years of life. Social Science & Medicine. 1982;16:1705-1708.

16) Bode L. Human milk oligosaccharides: prebiotics and beyond. Nutrition Reviews. 2009;67:S183-S191.

17) Bode L. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology. 2012;22:1147-1162.

18) Newburg DS, Walker WA. Protection of the neonate by the innate immune system of developing gut and of human milk. Pediatric Research. 2007;61:2-8.

19) Marcobal A, Barboza M, Froehlich JW, Block DE, German JB, Lebrilla CB, Mills DA. Consumption of human milk oligosaccharides by gut-related microbes. Journal of Agricultural and Food Chemistry. 2010;58:5334-5340.

20) Muñoz EC, Martin MJ, Prieto PA. 2′-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutrition Reviews. 2013;71:773-789.

21) Vasquez E, Barranco A, Ramirez M, et al. Effects of a human milk oligosaccharides, 2′-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents. The Journal of Nutritional Biochemistry. 2015;26:455-465.

22) Oliveros E, Ramirez M, Vazquez E, et al. Oral supplementation of 2′-fucosyllactose during lactation improves memory and learning in rats. The Journal of Nutritional Biochemistry. 2016;31:20-27.

23) Vazquez E, Barranco A, Ramirez M, et al. Dietary 2′-fucosyllactose enhances operant conditioning and long-term potentiation via gut-brain communication through vagus nerve in rodents. PLOS One. 2016;11:1-14.

24) Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Human Development. 1979; 3(1):79-83.

25) Lind NM, Moustgaard A, Jelsing J, Vajta G, Cumming P, Hansen AK. The use of pigs in neuroscience: Modeling brain disorders. Neuroscience & Biobehavioral Reviews. 2007; 31(5):728-51.

26) Dickerson JWT, Dobbing J. Prenatal and postnatal growth and development of the central nervous system of the pig. Proceedings of the Royal Society of London Series B, Biological Sciences. 1967;166:384-395.

27) Radlowski EC, Conrad MS, Lezmi S, Dilger RN, Sutton B, Larsen R, Johnson RW. A neonatal piglet model for investigating brain and cognitive development in small for gestational age human infants. PLoS One. 2014;9:e91951.

28) Pallotto EK, Kilbride HW. Perinatal outcome and later implications of intrauterine growth restriction. Clinical Obstetrics and Gynecology. 2006;49:257-269.

29) Resnik R. Intrauterine growth restriction. The American College of Obstetricians and Gynecologists. 2002;99:490-496.

30) Barry JS, Rozance PJ, Anthony RV. An animal model of placental insufficiency-induced intrauterine growth restriction. Seminars in Perinatology. 2008;32:225-230.

31) D'Inca R, Kloareg M, Guen CGL, Huërou-Luron IL. Intrauterine growth restriction modifies the developmental pattern of intestinal structure, transcriptomic profile, and bacterial colonization in neonatal pigs. The Journal of Nutrition. 2010;140:925-931.

32) Baserga M, Bertolotto C, Maclennan NK, et al. Uteroplacental insufficiency decreases small intestine growth and alters apoptotic homeostasis in term intrauterine growth retarded rats. Early Human Development. 2004;79:93-105.

33) Wang W, Degroote J, Ginneken CV, et al. Intrauterine growth restriction in neonatal piglets affects small intestinal mucosal permeability and mRNA expression of redox-sensitive genes. The FASEB Journal. 2016;30:863-873.

34) D'Inca R, Guen CGL, Che L, Sangild PT, Huërou-Luron IL. Intrauterine growth restriction delays feeding-induced gut adaptation in term newborn pigs. Neonatology. 2010;99:208-216.

35) Strauss RS. Adult functional outcome of those born small for gestational age: twenty-six-year follow-up of the 1970 British birth cohort. The Journal of the American Medical Association. 2000;283:625-632.

36) O'Keeffe MJ, O'Callaghan M, Williams GM, Najman JM, Bor W. Learning, cognitive, and attentional problems in adolescents born small for gestational age. Pediatrics. 2003;112:301-307.

37) American Academy of Pediatrics. Breastfeeding and the use of human milk [Policy statement]. Pediatrics. 2012;129:e827-e841.

38) CDC. Breastfeeding report card: Progressing toward national breastfeeding goals. National Center for Chronic Disease Prevention and Health Promotion. 2016;1-8.

39) Allen J, Hector D. Benefits of breastfeeding. New South Wales Public Health Bulletin. 2005;16:42-46.

40) American Academy of Pediatrics. Breastfeeding and the use of human milk. Pediatrics. 2005;115:496-506.

41) Qian L, Zhao A, Zhang Y, et al. Metabolomic approaches to explore chemical diversity of human breast-milk, formula milk and bovine milk. The International Journal of Molecular Sciences. 2016;17:2128.

42) Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. Pediatric Clinics of North America. 2013;60:49-74.

43) Boyd CA, Quigley MA, Brocklehurst P. Donor breast milk versus infant formula for preterm infants: a systematic review and meta-analysis. Archives in Disease and Childhood – Fetal and Neonatal Edition. 2007;92:F169-F175.

44) Institute of Medicine (US) Committee on the Evaluation of the Addition of Ingredients New to Infant Formula. Infant formula: evaluating the safety of new ingredients. National Academies Press. 2004;3:1-224.

45) Bode L. The functional biology of human milk oligosaccharides. Early Human Development. 2015;91:619-622.

46) Newburg DS. Are all human milks created equal? Variation in human milk oligosaccharides. Journal of Pediatric Gastroenterology and Nutrition. 2000;30:131-133.

47) Kunz C, Rudloff S. Compositional analysis and metabolism of human milk oligosaccharides in infants. Intestinal Microbiome: Functional Aspects in Health and Disease. 2017;88:137-147.

48) Bode L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. The Journal of Nutrition. 2006;136:2127-2130.

49) Vazquez E, Santos-Fandila SA, Buck R, Rueda R, Ramirez M. Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. British Journal of Nutrition. 2017;117:237-247.

50) Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT. The prebiotics 3'sialyllctose and 6'sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: evidence for effects on the gut-brain axis. Brain, Behavior, and Immunity. 2015;50:166-177.

51) Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants fed a lower calorie formula with 2′FL show growth and 2′FL uptake like breast-fed infants. Journal of Pediatric Gastroenterology and Nutrition. 2015;61:649-658.

52) Albermann C, Piepersberg W, Wehmeier UF. Synthesis of the milk oligosaccharide 2′-fucosylactose using recombinant bacterial enzymes. Carbohydrate Research. 2001;334:97-103.

53) Goehring KC, Kennedy AD, Prieto PA, Buck RH. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS ONE. 2014; 9:e101692.

54) Davis EC, Wang M, Donovan SM. The role of early life nutrition in the establishment of gastrointestinal microbial composition and function. Gut Microbes. 2017;8:143-171.

55) O'Sullivan A, Farver M, Smilowitz JT. The influence of early infant-feeding practices on the intestinal microbiome and body composition in infants. Nutrition and Metabolic Insights. 2015;8:1-9.

56) Liu Z, Roy NC, Guo Y, et al. Human breast milk and infant formulas differentially modify the intestinal microbiota in human infants and host physiology in rats. The Journal of Nutrition. 2016;146:191-199.

57) Lee SA, Lim JY, Kim BS, et al. Comparison of the gut microbiota profile in breast-fed and formula-fed Korean infants using pyrosequencing. Nutrition Research and Practice. 2015;9:242-248.

58) Yu ZT, Chen C, Kling DE, et al. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology. 2012;23:169-177.

59) Lewis ZT, Totten SM, Smilowitz JT, et al. Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. Microbiome. 2015;3:1-21.

60) Yu ZT, Chen C, Newburg DS. Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. Glycobiology. 2013;23:1281-1292.

61) Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG, Buck RH. Similar to those who are breastfed, infants fed a formula containing 2′-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. The Journal of Nutrition. 2016;146-2559-2566.

62) He Y, Liu S, Kling DE, Leone S, Lawlor NT, Huang Y, Feinberg SB, Hill DR, Newburg DS. The human milk oligosaccharide 2′-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. Gut. 2016;65:33-46.

63) He Y, Lawlor NT, Newburg DS. Human milk components modulate toll-like receptor-mediated inflammation. Advanced Nutrition. 2016;7:102-111.

64) Castillo-Courtade L, Han S, Lee S, Mian FM, Buck R, Forsythe P. Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. European Journal of Allergy and Clinical Immunology. 2015;70:1091-1102.

65) Miller ER, Ullrey DE. The pig as a model for human nutrition. Annual Review of Nutrition. 1987;7:361-382.

66) Mellor DJ, Cockburn F. A comparison of energy metabolism in the new-born infant, piglet, and lamb. Quarterly Journal of Experimental Physiology. 1986;71:361-379.

67) Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Human Development. 1979;311:79-83.

68) Widdowson EM. Intra-uterine growth retardation in the pig. I. Organ size and cellular development at birth and after growth to maturity. Biological Neonatology. 1971;19:329-340.

69) Buekens P, Delvoye P, Wollast E, Robyn C. Epidemiology of pregnancies with unknown last menstrual period. Journal of Epidemiology & Community Health. 1984;38:79-80.

70) Houle VM, Schroeder EA, Odle J, Donovan SM. Small intestinal disaccharide activity and ileal villus height are increased in piglets consuming formula containing recombinant human insulin-like growth factor-I. Pediatric Research. 1997;42:78-86.

71) Dilger RN, Johnson RW. Behavioral assessment of cognitive function using a translational neonatal piglet model. Brain, Behavior, Immunity. 2010;24:1156-65.

72) Elmore MR, Dilger R, Johnson R. Place and direction learning in a spatial T-maze task by neonatal piglets. Animal Cognition. 2012;15:667-76.

73) Fitz NF, Gibbs RB, Johnson DA. Selective lesion of septal cholinergic neurons in rats impairs acquisition of a delayed matching to position T-maze task by delaying the shift from a response to a place strategy. Brain Res Bull. 2008;77:356-60.

74) Conrad MS, Dilger RN, Johnson RW. Brain growth of the domestic pig (sus scrofa) from 2 to 24 weeks of age: a longitudinal MRI study. Developmental Neuroscience. 2012;34:291-98.

75) R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

76) Ameur A, Zaghlool A, Halvardson J, Wetterbom A, Gyllensten U, Cavelier L, Feuk L. Total RNA sequencing reveals nascent transcription and widespread co-transcriptional splicing in the human brain. Nature Structural & Molecular Biology. 2011;18:1435-40.

77) R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

78) Ameur A, Zaghlool A, Halvardson J, Wetterbom A, Gyllensten U, Cavelier L, Feuk L. Total RNA sequencing reveals nascent transcription and widespread co-transcriptional splicing in the human brain. Nature Structural & Molecular Biology. 2011;18:1435-40

79) Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biology. 2010;11:R25.

80) Robinson MD, McCarthy DJ and Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139-140

81) Leek JT and Storey JD. A general framework for multiple testing dependence. Proceedings of the National Academy of Sciences. 2008;105: 18718-18723.

82) Leek JT and Storey JD. Capturing heterogeneity in gene expression studies by 'Surrogate Variable Analysis'. PLoS Genetics. 2007;3:e161.

83) Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Research. 2015;43:e47.

84) Phipson, B, Lee, S, Majewski, IJ, Alexander, WS, and Smyth, GK. Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. Annals of Applied Statistics. 2016;10:946-963.

85) Law CW, Chen Y, Shi W, Smyth GK. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biology. 2014;15:R29.

86) Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series. 1995;B57:289-300.

87) Huber W, Carey VJ, Gentleman R, Morgan M. GenOrchestrating high-throughput genomic analysis with Bioconductor. Nature Methods. 2015;12:115.

88) Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protocols. 2009;4:44-57.

89) Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: path toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research. 2009;37:1-13.

90) Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. Nature Genetics. 2000;25:25-29.

91) Antonides A, Schoonderwoerd AC, Nordquist RE, van der Staay FJ. Very low birth weight piglets show improved cognitive performance in the spatial cognitive holeboard task. Frontiers in Behavioral Neuroscience. 2015;9:1-10.

92) Victoria CG, Barros FC, Horta BL, Martorell R. Short-term benefits of catch-up growth for small-for-gestational-age infants. International Journal of Epidemiology. 2001;30:1325-1330.

93) Lundgren EM, Cnattingius S, Jonsson B, Tuvemo T. Intellectual and psychological performance in males born small for gestational age with and without catch-up growth. Pediatric Research. 2001; 50:91-96.

94) Rehfeldt C, Kuhn G. Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. Journal of Animal Science. 2005;84:E113-E123.

95) Hanlon PR, Thorsrud BA. A 3-week pre-clinical study of 2′-fucosyllactose in farm pigs. Food and Chemical Toxicology. 2014;74:343-348.

96) Cohen E, Baerts W, van Bel F. Brain-sparing in intrauterine growth restriction: consideration for the neonatologist. Neonatology. 2015;108:269-276.

97) Nakagawa H, Iwasaki S, Kichikawa K, et al. Normal myelination of anatomic nerve fiber bundles: MR analysis. American Journal of Neuroradiology. 1998;19:1129-1136.

98) Eikenes L, Martinussen MP, Lund LK, et al. Being born small for gestational age reduces white matter integrity in adulthood: a prospective cohort study. Pediatric Research. 2012;72:649-654.

99) Hüppi PS, Dubois J. Diffusion tensor imaging of brain development. Seminars in Fetal Neonatal Medicine. 2006; 11:489-497.

100) Cook D, Kesner RP. Caudate nucleus and memory for egocentric localization. Behavioral and Neural Biology. 1988;49:332-343.

101) Roelants-van Rijn AM, van der Grond J, Stigter RH, de Vries LS, Groenendaal F. Cerebral structure and metabolism and long-term outcome in small-for-gestational-age preterm neonates. Pediatric Research. 2004;56:285-290.

102) Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nature Reviews: Genetics. 2002;3:662-673.

103) Grice LF, Degnan BM. The origin of the ADAR gene family and animal RNA editing. BMC Evolutionary Biology. 2015;15:4.